NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
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Project Director

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Volume 4 of 8

Neurochemical Protection of the Brain, Neural Plasticity and Repair

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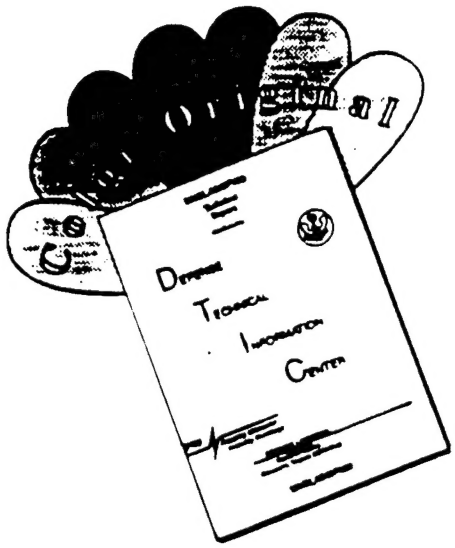

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ANIMAL USAGE

All use of animal tissues in this research was conducted under LSU IACUC protocol #1199 under the direction of Dr. Mark A. DeCoster. This protocol allows the use of 5 pregnant female Sprague/Dawley rats per week or 250 animals per year. To date, 155 animals have been used for this research.

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INTRODUCTION

The biologically active phospholipid platelet activating factor (1-*O*-alkyl-*sn*-glycero-3-phosphocholine, PAF), a phospholipase A_2 (PLA_2) product, is involved in excitatory neurotransmitter (i. e. Glutamate) release and synaptic plasticity (Bazan et al.,1993). Several physiological roles of PAF have been described, such as its ability to stimulate PC12 cell sprouting when present at low concentrations (Kornecki and Ehrlich, 1988), its stimulatory effect on glutamate release (Clark et al., 1992), and its involvement in the development of glutamate-mediated long-term potentiation (Del Cerro et al., 1990; Arai and Lynch, 1992; Wieraszko et al., 1993; Bazan et al., 1993), considered as a model of mechanisms underlying memory and learning in the brain (Teyler and DiScenna, 1984). As a consequence of neuronal cell injury, processes that normally modulate synaptic function are overactivated, leading to the accumulation of membrane lipid-derived second messengers such as 20:4 and PAF. These mediators of the cellular response to injury can lead to repair and/or to further cell damage and death (Bazan, 1994), depending upon their cellular origin and degree of neuronal activity impairment.

Overstimulation of glutamate transmission can lead to excitotoxic neuronal damage (Olney, 1986) and has been implicated in several chronic degenerative diseases in the CNS (Choi, 1988). Its neurotoxicity is attributed to Ca^{+2} influx into postsynaptic neurons, mediated by the *N*-methyl-*D*- aspartate (NMDA) receptor (Rothman and Olney, 1986, 1987; Choi, 1988). Overstimulation results in postsynaptic activation of PLA_2 by Ca^{2+} and PAF synthesis. PAF release can, in turn, exacerbate glutamate neurotransmission and NMDA-mediated neurotoxicity, leading to neuronal disfunction and eventually to cell death.

Because excess calcium influx and its subsequent sequestration by intracellular organelles, i.e. mitochondria and nucleus, play a key role in neuronal death (Nicotera et al., 1992), cortex and hippocampal cells in culture were used to further explore a) the cytotoxicity of PAF measured by lactate dehydrogenase release and by morphological evaluation of the

cells and b) calcium dynamics, monitored by a confocal laser scanning microscope in cells previously loaded with the fluorescent calcium indicator fluo-3.

BODY.

Previous work

Previous studies in this laboratory demonstrated that PAF displays a stimulatory effect on glutamate release (Clark et al., 1992), and that PAF could potentially be a retrograde messenger in long-term potentiation (Kato et al., 1994). To accomplish these effects PAF interacts with specific binding sites present in the CNS. The bioactivity of PAF towards receptors found in presynaptic membranes (Marcheselli et al., 1990) is to enhance excitatory amino acids release; the antagonist BN52021 blocks this effect (Clark et al., 1992; Bazan et al., 1993b).

Further studies summarized in the previous report (**year 1**), support a role for PAF as a trans-synaptic modulator of excitatory synaptic transmission. They demonstrate that PAF can cross the synapse and enhance the frequency of mepscs acting upon neighboring presynaptic neurons.

Objectives, year 2.

Below are listed the objectives of year 2 of this project:

A) Establish primary neuronal cell cultures from rat, including astrocytes cultures, whole cortical cultures, and hippocampal cultures.

B) Using primary neuronal cultures, establish neurotoxicity of excitatory amino acids (EAAs) and investigate the role of phospholipases and lipid mediators in neurotoxic action.

C) Establish techniques and technology for the measurement of signal transduction mechanisms in primary neuronal cultures.

D) Using the accomplishments of objective C, investigate the role of calcium ion flux in EAA neurotoxicity and potential modulation by lipid mediators.

Methods.

Cell Culture

Rat hippocampal cultures were produced using modifications of previously established techniques (Clark et al., 1992). Cell culture wells were coated with Matrigel (Collaborative Research) at a dilution of 1:3 with minimal essential medium (MEM) and stored in an incubator (37° C) until cell plating. Hippocampi were obtained from 1-3 day old rat pups using aseptic technique, and were pooled in Liebovitz's (L-15) medium with 0.04% bovine serum albumin (BSA) while under oxygenation, at 37° C during the dissection. Hippocampi were moved to a solution of 0.1% papain in L-15 + BSA for 20 minutes, then triturated with a Pasteur pipette. Single cells were isolated from tissue pieces, and viable cell yields were calculated using a hemocytometer and trypan blue exclusion. Cells were plated at a density of 500,000 cells/ml in MEM plus 10% fetal calf and horse serum supplemented with insulin, human transferrin and selenium and maintained in 37° C, 5% CO_2 incubators. After 24 hours, cultures were treated with 10^{-5} M cytosine arabinoside (AraC) to control astrocyte proliferation. After 4 days *in vitro* (DIV), AraC was removed and cells were moved to fresh media, as described above, but without serum. Hippocampal explant cultures were established as described above, except that tissues were not triturated into single dispersed cells, but rather separated into intact tissue pieces by a Pasteur pipette, and then plated onto culture wells. AraC was not added to explant cultures until 4 DIV, and was removed at 8 DIV. All experiments were carried out on cultures after at least 11 DIV. Primary rat cortical cultures and astrocyte cultures were prepared as previously described (DeCoster et al., 1992; DeCoster and Yourick, 1994). Assays for cytotoxicity measured lactate dehydrogenase (LDH) release and morphological evaluation of cells was as previously described (DeCoster et al., 1995).

Measurement of intracellular calcium.

For measurements of calcium dynamics in primary neuronal cultures, cells were loaded with the fluorescent calcium indicator fluo-3, as previously described (DeCoster et al., 1992a). Calcium dynamics were monitored with a Noran Instruments Odyssey XL confocal laser scanning microscope. Digitized images were captured on a Silicon Graphics Indy workstation, and calcium changes in the cells analyzed using Noran Intervision software on Indy workstations.

The Noran Instruments scanning laser confocal microscope has been set up at the Neuroscience Center for approximately one year. Significant upgrades have been added to both the software and hardware capabilities since the system was first purchased. These upgrades and their importance are briefly summarized below:

(A) A third photomultiplier tube (PMT) and a transmitted light detector have been added to the system. The third PMT will allow us to detect three fluorescent probes simultaneously on the system, or one probe and the ratio of two other probes. The transmitted light detector allows us to capture visible light images of cells and tissues and thus superimpose fluorescent probe images onto these visible light images.

(B) As part of a Louisiana intrastate grant, we have obtained a second Silicon Graphics Indy workstation for our imaging lab. We have purchased a second copy of Noran's Intervision analysis software for this second Indy so that it may serve as another site for data analysis. A 24 bit video card and an external hard disk were also purchased for this workstation so that it would run the necessary Intervision software files.

(C) Both the Noran Instruments Indy workstation and the second Indy have been upgraded in random access memory to more efficiently and more quickly analyze data files. A 1.2 Gigabyte optical disk device was purchased for more efficient data storage.

Results.

A). Primary neuronal cultures consisting of whole cortex, astrocyte, and hippocampal preparations were successfully established. These cultures were characterized by immunocytochemistry and microscopic examination. All neuronal cultures were prepared under LSU IACUC protocol #1199 under the direction of Dr. Mark A. DeCoster.

B). The cytotoxic sensitivity of established neuronal cultures to EAAs such as glutamate and kainate was evaluated. In both cortical and hippocampal cultures, LDH release is being used as the assay for cytotoxicity. In cortical cultures, a 45 minute exposure to EAAs at room temperature was found to be optimal (Kolko et al., 1995 and 1995a), which is consistent with previously established techniques (DeCoster et al., 1992 and 1995) (**Figure 1**).

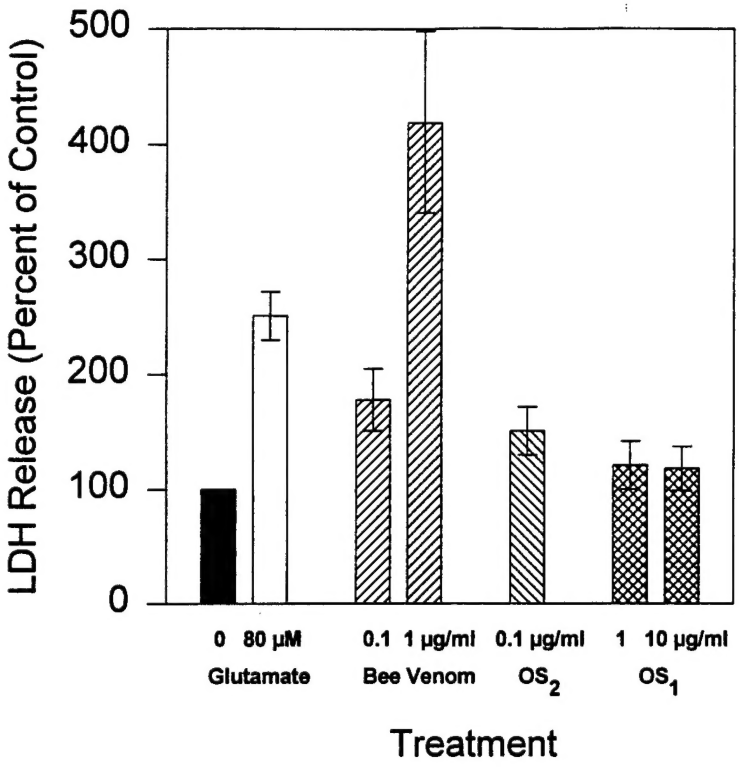

Figure 1. Toxicity of Phospholipases A_2 and glutamate to primary rat cortical neurons. Rat primary cortical neurons were exposed to the indicated treatments for 45 minutes at room temperature in Locke's solution without magnesium. This solution was then replaced with Locke's plus magnesium and the cells placed at 37° C in a 5% CO_2 incubator overnight until assaying for LDH release as indicated in methods. Data are normalized to LDH release of control cultures (treated only with Locke's solution), and show representative averages from one of multiple experiments, with standard deviations shown.

Hippocampal cultures have been found to be more sensitive to these treatments than cortical cultures, so we are exposing these cultures to EAAs at room temperature for only 15 minutes (Mukherjee et al., 1995 and 1995a) (**Figure 2**). Thus far, we have found that two phospholipases A_2 (PLA_2) specific for neuronal binding are toxic to the cortical cultures, whereas a PLA_2 specific for muscle is not toxic to the neuronal cultures (Kolko et al., 1995 and 1995a).

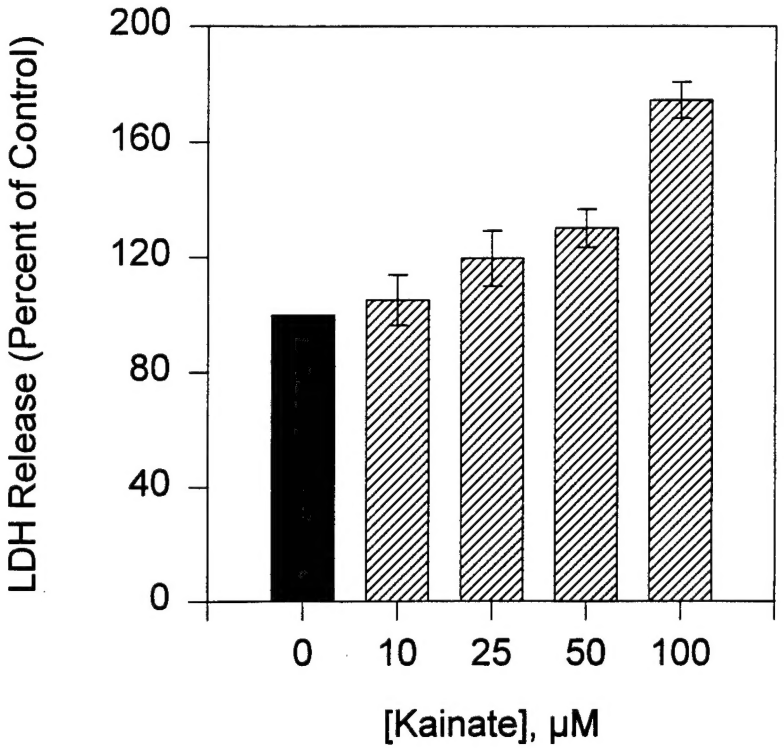

Figure 2. Toxicity of kainate to primary rat hippocampal neurons. Rat primary hippocampal neurons were exposed to the indicated concentrations of kainate for 15 minutes at room temperature in Locke's solution without magnesium. This solution was then replaced with MEM plus Earle's salts and the cells placed at 37° C in a 5% CO_2 incubator overnight until assaying for LDH release as indicated in methods. Data are normalized to LDH release of control cultures (treated only with Locke's solution), and show averages of multiple experiments; n=6-13 for each point with Standard error of the mean (SEM) shown.

C). As described in the methods section, the Noran Instruments confocal laser microscope system has been our tool for measuring calcium dynamics in cell culture. While we have been making significant technical upgrades to this instrumentation, we have nevertheless successfully used this technology to obtain images showing changes in neuronal calcium after the addition of lipid mediators (**Figure 3**).

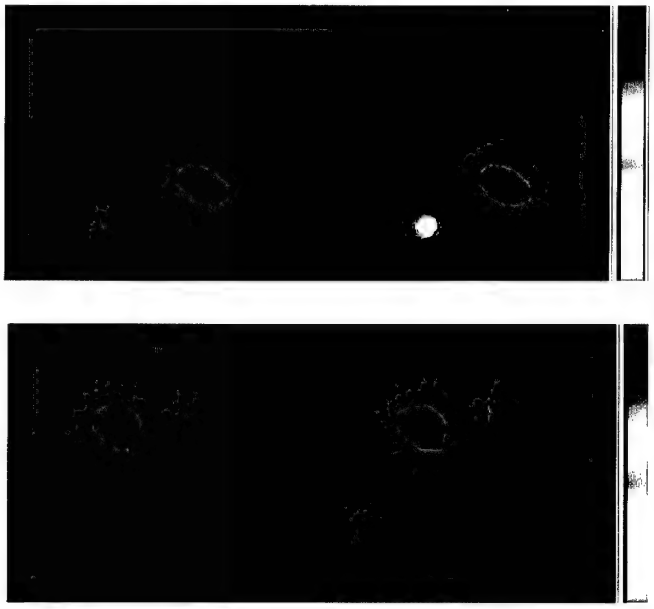

Figure 3. Intracellular calcium concentration ($[Ca^{2+}]_i$) modulated by PAF in primary rat hippocampal cultures. Cultures loaded with fluo-3 were scanned using a confocal microscope imaging system (60X water immersion lens) before (top left) and after addition of PAF (top right); addition of BSA alone (bottom left and right) showed no changes in $[Ca^{2+}]_i$.

D). Using the accomplishments of result 3 above, we are now able to monitor calcium dynamics in cortical and hippocampal cultures in response to EAAs and lipid mediators. Thus far, we have found that although the secretory PLA_2s are highly toxic to the cortical neurons compared to glutamate, these PLA_2s do not consistently cause a large calcium influx as does glutamate, but rather often decrease basal calcium levels (Kolko et al., 1995 and 1995a). In the hippocampal cultures, similar responses to glutamate have been observed as in the cortical cultures. We have found that in the hippocampal cultures, short-term pretreatment (30 minutes) with PAF or methyl carbamyl PAF (mcPAF), but not with lysoPAF or the carrier bovine serum albumin (BSA), potentiates calcium responses to subsequent submaximal stimulus by glutamate. Acute treatment of hippocampal neurons with PAF or mcPAF caused an average increase of intracellular calcium in these cells, and induced increased basal calcium oscillatory activity in some; these effects were not observed in cells treated with lysoPAF or

BSA. However, in PAF and mcPAF-treated hippocampal neurons, overnight treatment with these same compounds resulted in less sensitivity in calcium flux to submaximal concentrations of glutamate added to the cells the next day. However, neurons treated with lysoPAF and BSA were more responsive in calcium flux to these glutamate additions. We have hypothesized that responses indicating an increase in calcium flux, demonstrated by acute PAF addition, may continue when cells are chronically exposed, causing EAA release in the cultures and thus desensitizing the cells to glutamate on the subsequent day (DeCoster et al., 1995a and 1995b). The acute effects of PAF on hippocampal neuronal calcium dynamics are summarized below in **Figure 4.**

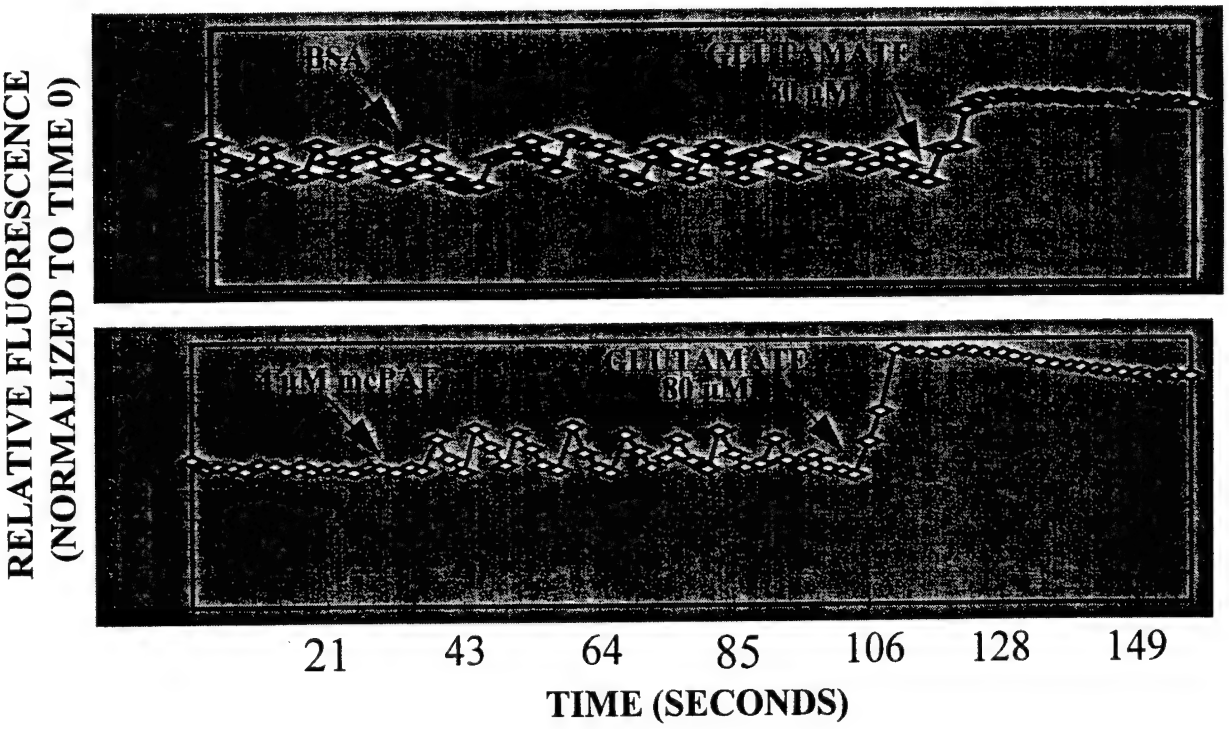

Figure 4. Induction of $[Ca^{2+}]_i$ oscillations by mcPAF in hippocampal neurons. Rat primary hippocampal neurons were loaded and scanned as described in Fig. 3. mcPAF in BSA (bottom panel) or BSA alone (top panel) was added to neurons as indicated by arrows and $[Ca^{2+}]_i$ dynamics were then monitored. Towards the end of each scan, glutamate was added

to elicit a positive response from cells. Once added to cultures, all substances remained in the culture well. Relative fluorescence has been normalized to the values as time 0, and data were collected every 2 seconds during scanning.

SUMMARY AND CONCLUSIONS

Primary cultures of whole cortex and hippocampal neurons have been established and the sensitivity to EAAs has been characterized. We used two neuronal specific and one muscle specific secretory PLA_2 to investigate the toxicity of these compounds to cortical neurons. The neuronal specific PLA_2s were found to be toxic, as was also a different secretory PLA_2 in the same culture model (DeCoster et al., 1994a; Clapp et al., 1995). Surprisingly, the neuronal calcium response to toxic concentrations of these PLA_2s was a decrease in basal calcium levels, compared to the large increase in calcium levels observed upon addition of glutamate.

Primary hippocampal cultures were found to be more sensitive to neurotoxic conditions than were whole cortical cultures. Acute treatment with PAF was found to induce an average increase in calcium in hippocampal neurons, and in some cells caused more calcium oscillations. Short-term pretreatments also appear to potentiate subsequent neuronal responses to submaximal concentrations of glutamate, as measured by calcium levels. In contrast, long-term (overnight) treatments with PAF desensitizes neurons to submaximal glutamate calcium responses.

Major technological advances have been made in our calcium and confocal imaging capabilities by significant software and hardware upgrades. These upgrades should allow a more in-depth analysis of lipid mediators in neuronal injury at the level of the cell.

Research plan for the upcoming year.

1. We will continue to characterize the sensitivity of cortical and hippocampal cultures to EAAs with an emphasis on modulation by lipid mediators. Namely, does PAF alter the neurotoxic potential of EAAs? Do PAF receptor antagonists change the neurotoxic potential of these EAAs?

2. We will investigate the ability of PAF antagonists to alter the calcium dynamics, which we have reported is an effect of PLA_2 and PAF. Do these antagonists change basal calcium oscillations, the apparent augmentation by PAF, or the decrease in basal calcium levels by PLA_2

addition?

3. Techniques and technology for electrophysiological recordings of neuronal preparations, with the intent of recording from neuronal cell cultures, will be established with the postdoctoral assistance of Dr. David Linn. An alternative approach for this objective includes development of hippocampal explant cultures as an option to single-cell and slice preparations. It is expected that neuronal explant cultures will serve as an intermediate between dispersed cell cultures and slice preparations. These explant cultures will most likely be thinner in the Z-axis than slice preparations, which will be an advantage during confocal scanning, and will allow more of a 3-dimensional cellular array of neurons and astrocytes than is observed for traditional cell cultures. There may be important neuronal cell types that do not thrive after cell dispersion techniques that will thrive in the explant culture. Also, these approaches will be developed with the ultimate goal of developing an electrophysiological recording with simultaneous calcium imaging from the confocal microscope.

Abstracts and publications from this work.

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October 4, 1995

TITLE: NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR.

CHAPTER: NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR.

THE KINDLING MODEL OF EPILEPTOGENESIS AND SYNAPTIC PLASTICITY.

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INTRODUCTION

Status epilepticus is associated with cell loss in hippocampal areas such as CA1, CA3 and the hilus of the dentate gyrus and in the neocortex and subcortical areas (Hauser, 1983). Similar hippocampal sclerosis has been reported (Cavazos et al., 1994) during kindling, an animal model of epilepsy in which a series of initially subconvulsive stimulations leads to progressive intensification of seizure activity that culminates in generalized motor activity (Goddard, 1967; McNamara et al., 1992). Over stimulation of glutamate release and its subsequent interaction with postsynaptic NMDA receptors results in excitotoxic neuronal damage (Olney, 1986). Excitotoxicity has been involved in degenerative diseases of the CNS (Choi, 1988), as well as in ischemia and seizures (Choi and Rothman, 1990). NMDA antagonists inhibit kindled seizures (McNamara et al., 1988; Löscher and Hönack, 1991). Recently, Kraus et al., (1994) have reported that kindling induces the long-lasting expression of a novel NMDA receptor in the CA3 region of the hippocampus that may contribute to enhanced glutamate-NMDA synaptic activity and hyperexcitability. However, the molecular mechanisms that lead to enhanced glutamate release and to cell death and abnormal synaptic reorganization are not known.

Using the rat model of rapid kindling, a primary objective of this project is to elucidate the role of second messenger platelet activating factor (1-*O*-alkyl-*sn*-glycero-3-phosphocholine, PAF) in epileptogenesis. Previous studies have shown that the biologically active phospholipid PAF, a phospholipase A_2 (PLA_2) product, is involved in synaptic plasticity (Bazan et al., 1993; Kornecki and Ehrlich, 1988), has an stimulatory effect on glutamate release (Clark et al., 1992), and is a potential retrograde messenger in long-term potentiation (Kato et al., 1994). Although low PAF concentrations can modulate cell function, at higher concentrations it may become an endogenous neurotoxin contributing to synaptic changes and brain damage. We have recently discovered that PAF, acting at an intracellular site in neural cells (Marcheselli et al., 1991), leads to the expression of immediate early genes (IEG, Bazan, 1994). Some of these genes (i.e. *Zif*-268) encode transcription factors that will trigger a cascade of gene expression. These, in turn, may initiate either neuronal cell damage and death or repair-regenerative responses (Bazan et al., 1993; Bazan, 1994).

COX-2, a recently cloned gene, is a member of the immediate-early gene family (DeWitt et al, 1988; Kujubu and Herschman, 1991.). COX-2, which encodes one subtype of the enzyme Prostaglandin Synthase-2 (PGHS-2, Tis-10), is rapidly induced in the neural tissues by a different sort of stimuli, including PAF (Bazan et al., 1994). In contrast, Prostaglandin Synthase-1, or PGHS-1, also called COX-1, is a constitutively expressed enzyme (DeWitt and Smith, 1988). PGHS-2 and the constitutive PGHS-1 may contribute to eicosanoid production from free AA (Hershman, 1994), the latter under normal basal conditions and the former when synaptic activity is overstimulated (i. e. after ischemia, seizures), thus contributing to the synthesis of other mediators of neuronal damage.

The use of the antagonist BN50730, which competes with PAF for intracellular binding sites involved in PAF-induced gene expression (Bazan, 1994), will allow us to study and correlate PAF involvement during kindling in **a-** evoked behavioral seizures, **b-** EEG recording, **c-** early genes expression, and **d-** morphological alterations in the hippocampus (i.e. neuronal loss, mossy fiber axon sprouting, and synaptic reorganization).

BODY.

Previous work.

During the first year of this project we set up a computer driven system for fast hippocampal kindling of the rat. This model has the advantage that kindling is accomplished over a 21-day period. Moreover, as many as 8 animals can be stimulated and electroencephalographically recorded at one time. Also, we developed the surgical procedure for infusion of PAF antagonists via a cannulae implanted in the left ventricle and attached to a mini osmotic pump placed in the mid-scapular region of the back. Using this fast kindling paradigm, we began our studies about PAF involvement in activation of immediate early genes (IEG) during kindling, as detailed below.

Objectives, year 2.

A) The fast kindling model and PAF involvement in IEG expression, cellular plasticity, and neuronal cell damage. This model was used to follow the effect of the PAF antagonist BN50730 on the establishment and maintenance of kindled seizures and to correlate this result

with its effect on IEG expression.

A-1) *Behavioral classification*: All seizures in control (CSF and DMSO treated) and experimental (BN50730 treated) animals were classified according to the scale described by Racine, *1972:* **class 1**, wet dog shakes; **class 2**, facial clonus; **class 3**, forelimb clonus, head bobbing; **class 4**, rearing; **class 5**, rearing with loss of balance.

A-2) *EEG recording analysis during kindling in sham (CSF and DMSO) versus BN50730 treated rats*: Analysis was done on the animals' electroencephalographic records to determine the lengthening of after-discharge duration (ADD) throughout the kindling process. ADD was determined by timing the duration that the animals EEG was greater than 2X the baseline EEG following stimulation.

A-3) *Comparison of computer versus manually analyzed ADD data:* we have set up a EGAA computer scope for automatic analysis of ADD. This procedure will allow a faster, more accurate and efficient way to analyze the EEG seizure record generated from hundreds of animals used in these studies.

A-4) *IEG expression (TIS-8, TIS-10 or COX-2)*: this work is in progress.

A-5) *Histological analysis*: Because PAF action in the CNS can mediate either plasticity responses and/or neuronal damage leading to cell death, we began to explore its role during kindling in mossy fiber sprouting (as a plasticity response) and hippocampal sclerosis (as a neural damage). These studies were done in control (CSF and DMSO) and experimental (BN50730) treated rats following the experimental protocol of kindling and a subsequent series of class 5 seizures as detailed in Methods. Analysis of the data are currently in progress.

B) Immediate early gene expression after a single ECS. As mentioned in the original proposal, the kindling studies were complemented by following early gene expression in the hippocampus after a single, maximally convulsive ECS from control and BN50730-treated rats.

B-1) *Northern Blot analysis.* The mRNA expression of the IEG: *Zif*-268 and COX-2 (Tis-10, Cyclooxigenase-2, or PGSH-2) genes was followed as a function of time after 1-ECS in the hippocampus of control- BN50730- and dexamethasone-treated rats.

B-2) *Nuclear run-off or in vitro transcription assay:* This experimental approach was

followed to further explore if changes in IEG mRNA levels induced by 1-ECS is related to a specific activation of gene expression or to the action of mRNA binding proteins that stabilize the message.

Methods

Kindling. Male Sprague Dawley rats (275-300 g) were anesthetized with Xylazine/Ketamine (100 mg/Kg each) and stereotaxically implanted with a stainless steel electrode in the right ventral hippocampus (lateral 0.49 cm, posterior 0.36 cm, depth 0.50 cm, tooth bar at +0.50 cm). Animals were kindled following the procedure detailed in **Figure 1**.

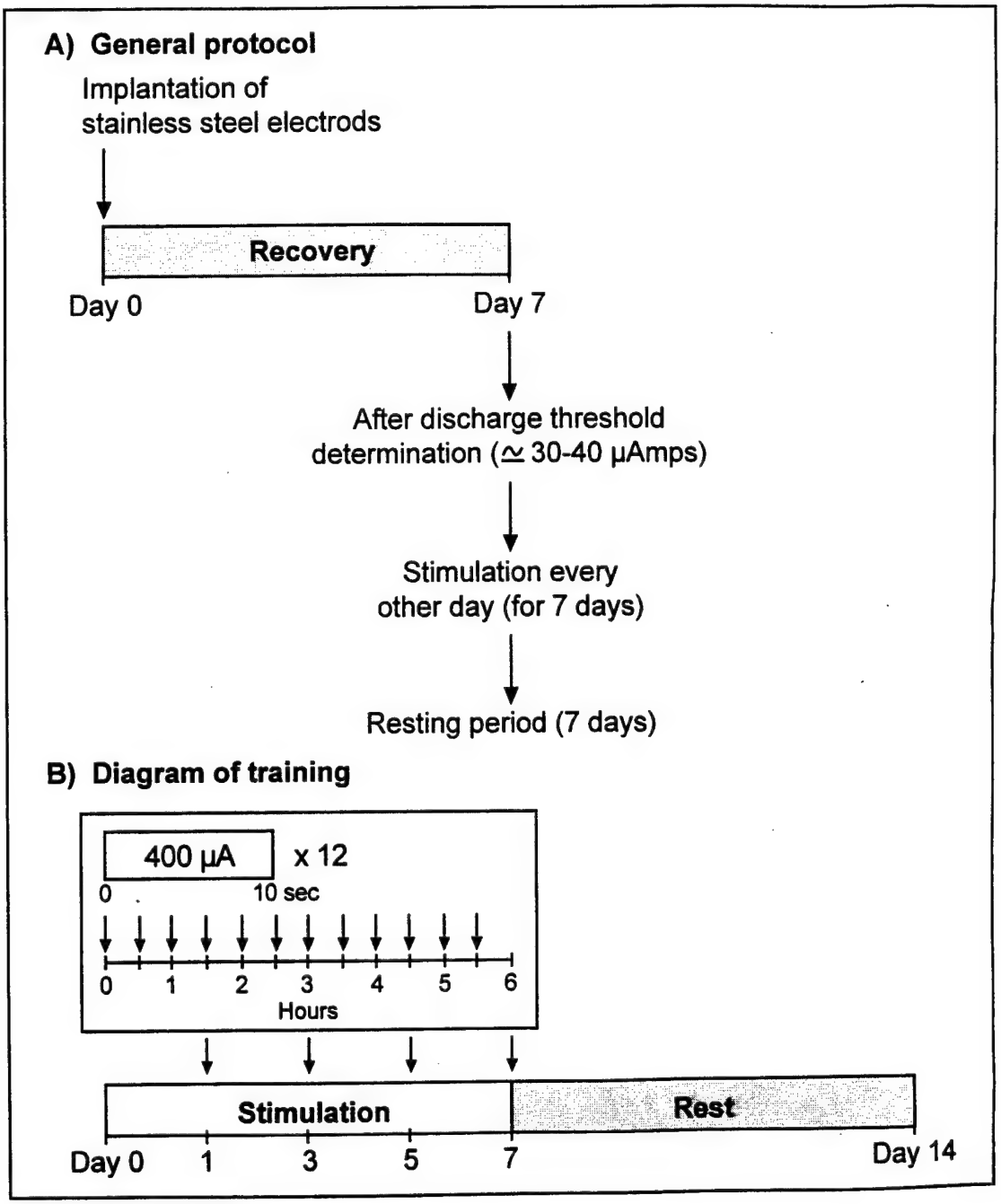
Animal treatment with PAF antagonist BN50730 during kindling. A stainless steel cannulae was placed in the lateral ventricle contralateral to the site of electrode implantation (lateral 0.25 cm, posterior 0.08 cm, depth 0.30 cm, tooth bar +0.50 cm). A mini-osmotic pump filled with CSF was attached to the cannulae with polyethylene tubing and placed in the mid-scapular region of the back. Seven days following surgery the mini-osmotic pump was replaced and an external reservoir made of polyethylene tubing and filled with BN50730 (6 μg/μl) , CSF, or DMSO was implanted in the animal between the pump and the cannulae. Animals were given three days to recover before initiation of kindling. The rate of infussion was 0.5 μl/h and the final dose of BN50730 was 72 μg per day.

Statistical analysis of the data. A two level factorial analysis of variance (ANOVA) was performed on the behavioral and A. D. D.analysis of the data (Freund and Littel, 1981). For both studies significant differences were observed between treatments (DMSO and BN50730 infusion, $p<0.0001$).

General histological procedures: 1- perfusion, fixation, and embedding.

Rats were anesthetized with an i.p. injection of 0.8-1.1 ml of ketamine and rompun (9:1) and then prepared for fixation by transcardial perfusion. Physiological saline (0.9% NaCl containing 5 mM EGTA) was first applied for 5 min, followed by 0.1% sodium sulfide in 0.15 M Sorensen phosphate buffer for 7 min. Fixative, consisting of 2.5% formaldehyde and 2.5% glutaraldehyde in 0.15 M Sorensen buffer, was then added for 10 minutes. All solutions were perfused at a pressure of 120 mm Hg. The brain was then removed and the hippocampal regions

Figure 1: Experimental design for the fast kindling paradigm accomplished over a 21-day period.



isolated and placed in fresh, cold fixative overnight. Tissue was cut into 1-2 mm thick slices and rinsed in Sorensen buffer for 3 x 15 minutes and dehydrated to 100% ethanol in 20% steps. Some samples were cleared in several changes of toluene for 2-3 days, infiltrated with paraffin, and embedded for thick (10 μm) paraffin sectioning. Other samples were placed in acetone, infiltrated with Epon-Araldite plastic, and embedded in molds for thin (1 μm) plastic sectioning. The methods outlined in Danscher (1981) were followed.

2- Cresyl violet staining for general tissue surveys and nuclear counts.

Paraffin sections (10 μm thick) were affixed to subbed slides. Sections were deparaffinized and hydrated to distilled water. They were then stained for 20 minutes in the cresyl violet working solution, consisting of 10 ml cresyl violet solution (0.2 g cresyl violet in 150 ml distilled water) in 100 ml of buffer solution (94.0 ml 0.1 N acetic acid and 6 ml 0.1 M sodium acetate). Following a brief rinse in distilled water, slides were coverslipped in Aqua-Poly/Mount. Generally, the procedures outlined in Humason (1972) for Nissl substance were followed.

3- Sulfide silver demonstration of zinc concentrated in nerve terminals (Timm's stain).

Plastic sections (1 μm thick) were collected onto drops of distilled water and dried onto very clean glass slides. After coating slides by dipping in 0.5% gelatin, sections were stained for sulfide silver to demonstrate regions of zinc accumulation within nerve fibers of the hipopocampus (Timm staining) according to procedures outlined by Danscher (1981). The following solutions are used: *A. Protecting colloid.* Gum arabic (50% solution). *B. Citrate buffer.* Citric acid (25.5 g), sodium citrate (23.5 g), water (100 ml total). *C. Reducing agent.* Hydroquinone (0.85 g) in 15 ml water. *D. Silver ions.* Silver lactate (0.11 g) in 15 ml water. *Developer solution.* A, 60 ml; B, 10 ml; C, 15 ml; D, 15 ml. Slides were placed flat in moist chamber staining dishes, sections covered with developer, and dishes placed into a 26° C incubator for 30-150 minutes. After developing, slides were rinsed 3 x 5 minutes in distilled water, then rinsed in 40° C running tap water until the protective gelatin coat had been removed. All above steps were done in darkness or very dim light. Following a final rinse in distilled water, plastic sections were coverslipped with Aqua-Poly/Mount. No counter staining occurred. Instead, Nomarski DIC optics were employed.

Electroconvulsive shock (ECS) and PAF antagonists pretreatment.

Animals, albino Sprague Dawley rats (250 to 300 g body weight) were used. An incision on the scalp along the mid line to expose the skull was done in animals under ether anesthesia. Using a 1mm dental burr drill bilateral holes were made without penetrating the dura. After a 24 hours recovery animals were anesthetized with ether and PAF antagonist or vehicle were injected intracerebroventricularly (icv) using a Hamilton syringe. The PAF antagonist BN50730 was solubilized in DMSO, and injections of 2 µl per site were performed 30 minutes before ECS, delivering 30 µg compound per animal. The use of a calibrated canulae attached to the needle of a Hamilton syringe, permits injections to accurately reach a deepness of 4.5 mm. ECS (100V square wave direct current, 0.5 ms pulse duration, stimulation rate 150 p.p.s., train duration 750 ms) was delivered with a Grass S48 stimulator via a pair of platinum electrodes placed under the skin at each side of the forehead. The animals were left to recover, and killed at different times after ECS (0, 1, 2, 6, 12, and 24 hours). Sham operated are animals (controls) that were handled as experimental ones except for the electrical discharge.

Tissue dissection, RNA extraction and Northern blot analysis.

Brain tissue was rapidly dissected on an ice cold dissection board, and brain hippocampus rapidly removed. Total RNA from brain regions was isolated following the guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). Tissue was homogenized with a Polytron type homogenizer, in 4 ml buffer containing 4 M Guanidine Thiocyanate, 25 mM sodium citrate, 0.5 % n-lauroyl sarcosine, and 0.1 M mercaptoethanol, pH 7.0. After precipitation the just purified RNA extract was resuspended in DEPC (diethyl-pirocarbonate) treated water, and an aliquot was quantified by spectrophotometric detection in a range 300 to 220 nm. Gel electrophoresis of RNA (5 µg per lane) was performed under denaturing conditions on a 1.2 % agarose gel. RNA was transferred to Hybond-N^+ nylon Membranes (Amersham, Arlington Heights Illinois), followed by hybridization at 42 °C with ^{32}P-labeled DNA probes for *Zif*-268, Tis-10, c-*fos*, *jun*-b, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ^{32}P-DNA probes were obtained by random primer extension from cDNA inserts of c-*fos* (Curran et al., 1987), zif-268 (Millbrandt, 1987), Tis-10 (Kujubu, and Herschman, 1991) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH),(ATTC # 57090), (Tso et al., 1985). Autoradiography or phosphor-image quantification were performed on a

Biorad® instrument.

Isolation of nuclei from hippocampal tissue.

All operations were at 4°C on wet ice using proteolytic enzyme inhibitors phenylmethylsulfonyl fluoride (PMSF, Sigma; 1 mM), aprotinin (Sigma; 0.05 ug/ul) and leupeptin (Sigma, 0.025 ug/ul). The hippocampal tissue mass was weighed (typically 0.2 - 1 mg wet weight) and a Kontes cordless hand held mini homogenizer (Cat.No. 749540) was used to homogenize the cells in ~500 ul of Dubelccos Phosphate Buffered Saline (PBS) containing 1 mM PMSF and 0.05 ug/ul aprotinin, in a 1.5 ml Eppendorf vial, until suspension was completely homogeneous. This suspension was pelleted at 4°C by centrifugation at 1400 x Gav for 10 minutes (3500 rpm in an Eppendorf 5403 centrifuge) to get rid of blood vessels, extracellular debris, etc., and after carefully removing the supernatant the pellet was gently resuspended in 500 ul of NUCLEI PREPARATION BUFFER (20 mM HEPES; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1mM DTT; 0.5 mM PMSF; 1% (v/v) aprotinin; 0.6%, v/v Nonidet P-40). 500 ul of buffer was required per single hippocampus, in each 1.5 ml Eppendorf tube. Nuclei were pelleted at 2600 x Gav (5 Krpm in an Eppendorf 5403 centrifuge) for 4 minutes and supernatant was removed. An aliquot of the suspension was stained with 0.1% crystal violet and observed under the microscope. Samples were kept frozen at -20°C until use.

Nuclear run-off or *in-vitro* transcription assay:

Nuclear samples were resuspended in 100 µl 2X reaction buffer containing 0.1 M ATP, 0.1 M CTP, 0.1 M GTP and 0.1 M DTT, the reaction started by the addition of 12.5 µl of ^{32}P-UTP (800 Ci/mmol, 40 mCi/ml) and samples incubated for 30 minutes at 30°C with shaking. 0.6 ml of DNAase I solution in HSB buffer was added, followed by an additional 5 minutes incubation at

30°C with shaking. Following the addition of 200 µl of 0.5 M Tris-Cl pH 7.4, 0.125 M EDTA, 5% SDS, and 10 µl of proteinase K solution, samples were incubated at 40 °C for 30 minutes and then 10 µl of 10 mg/ml tRNA carrier was added .Samples were mixed carefully and extracted with 1 ml of buffered phenol:chloroform:isoamyl alcohol (25:24:1, by vol.), and centrifuged 10 minutes at 6000 RPM at 4 °C. The aqueous layer was collected, and the phenolic phase was washed with 2 ml TE buffer pH 8.0. The aqueous layers were combined and the phenolic phase

discarded. 3 ml 10% TCA, 60 mM sodium pyrophosphate was added to the collected aqueous layers (about 3 ml/sample) and samples were incubated for 30 minutes in ice. TCA precipitates were filtrated through Millipore type HA (0.45 μm) filters to remove unincorporated nucleotide, and filters were washed 3 times with 10 ml of ice cold 5% TCA, 30 mM sodium pyrophosphate. Filters were placed into scintillation vials and incubated with 1.5 ml DNAase I buffer, and 37.5 μl of DNAase I (1 mg/ml) for 30 minutes at 37 °C. The reaction was quenched by addition of 45 μl of 0.5 M EDTA, and 68 μl of 20% SDS and samples heated to 65 °C for 10 minutes to elute the RNA. The eluate was removed and collected in a centrifuge tube. 0.75 ml of 10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% SDS was added and samples incubated at 65 °C for 10 minutes. The eluate was removed and saved. To the about 3 ml filter eluate, 4.5 μl of proteinase K (20 mg/ml) was added and samples incubated at 37 °C for 30 minutes. RNA was extracted with 3 ml of buffered phenochloroform:isoamyl alcohol (25:24:1, by vol.), mixed and centrifuged at 6000 RPM for 10 minutes at 4 °C. The aqueous layer was removed and saved. To the aqueous phase 0.75 ml of 1 M NaOH was added, followed by incubation on ice for 10 min. The reaction was quenched with 1.5 ml of 1 M HEPES (acid free). After adding to the tubes 0.53 ml of 5 M NaOAc. and 14.5 ml 95% ethanol, RNA was precipitated by overnight incubation at - 20 °C, or 30 minutes in dry ice ethanol. Tubes were centrifuged at 6000 RPM for 10 minutes at 4 °C, liquids removed very carefully, to avoid removing precipitates, and pellets dried under a vacuum for 1 hour. RNA was resuspend in 100 μl of 10 mM TES pH 7.4, 0.2% SDS, 10 mM EDTA (short incubation at 42 °C will accelerate the solubilization). 2 μl alicuots were counted to calculate total activity. Samples were diluted as necessary to reach a final count of 2.5 X 10^6 dpm in 300 μl solution. In 7 ml scintillation vials 300 μl RNA solution was pipeted, then 300 μl of TES/NaCl solution (10 mM TES pH 7.4, 0.2% SDS, 10 mM EDTA, 600 mM NaCl). A slot-blot strip with target plasmid cDNAs was placed into each vial, with application side facing the inside of the vial and samples were incubated at 42 °C for 48 hours, in a rotatory incubator. Strips were washed for at least 1 hour in 2 X SSPE, 0.1% SDS, at 65 °C and exposed to phosphor immager plate overnight, or X-ray film.

Results

A-1) The behavioural analysis of seizures during kindling is shown in **Figure 2.** Although animals in both treatment groups tend to exhibit class 5 seizures, the animals treated with BN50730 express fewer class 5 seizures and develop these class 5 seizures later in the kindling process than the control animals do.

-No statistical differences were noted during the first day of stimulation with respect to behavioral scores.

-In animals treated with the PAF antagonist, BN-50730, there is a statistically significant decrease in the severity of the seizures expressed throughout the kindling process when compared to controls. In addition, the rapidly induced short-lasting increase in seizure severity (described by Lothman and Williamson, 1994) is statistically different in the animals treated with BN-50730 when compared to controls.

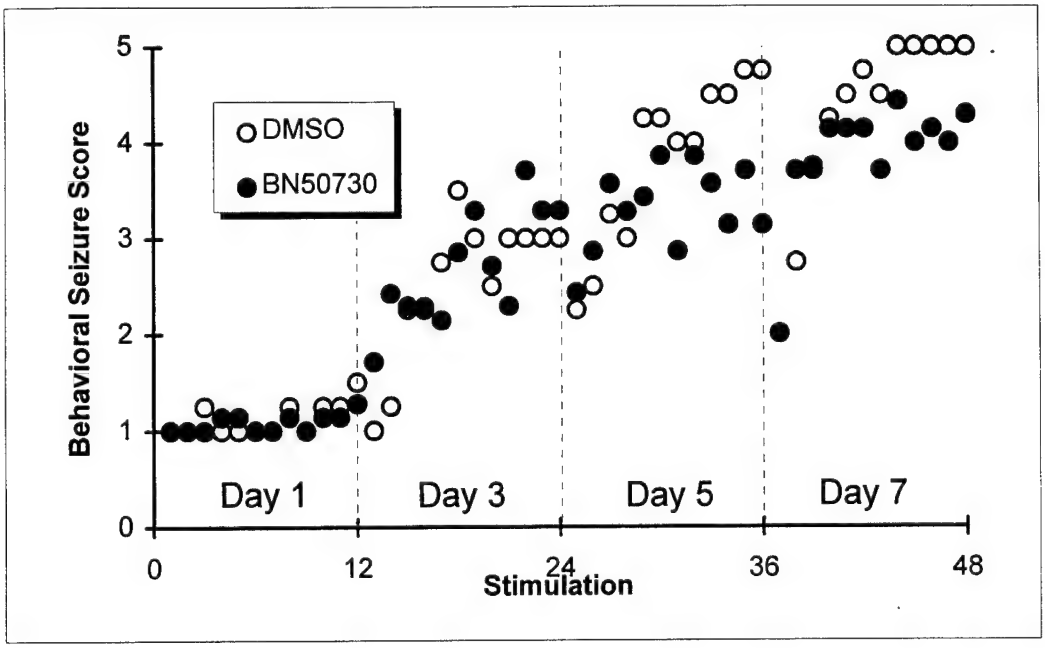


Figure 2 Behavioral analysis of seizures during kindling. Effect of BN50730 treatment. Significant differences were observed between treatments (ANOVA, $p<0.0001$)

A-2) The electroencephalographic analysis of after discharge duration (ADD) is shown in **Figure 3**. In animals treated with the PAF antagonist, BN-50730, there is a statistically significant decrease in the lengthening of the after discharges throughout the kindling process when compared to controls. In addition, the rapidly induced short lasting lengthening of the after discharge durations are significantly reduced in the animals treated with BN-50730 when compared to controls.

-Statistical differences started to emerge on the second day of stimulation and continued throughout the rest of the protocol.

-During the third and fourth day of stimulation the differences between treatment groups widened greatly.

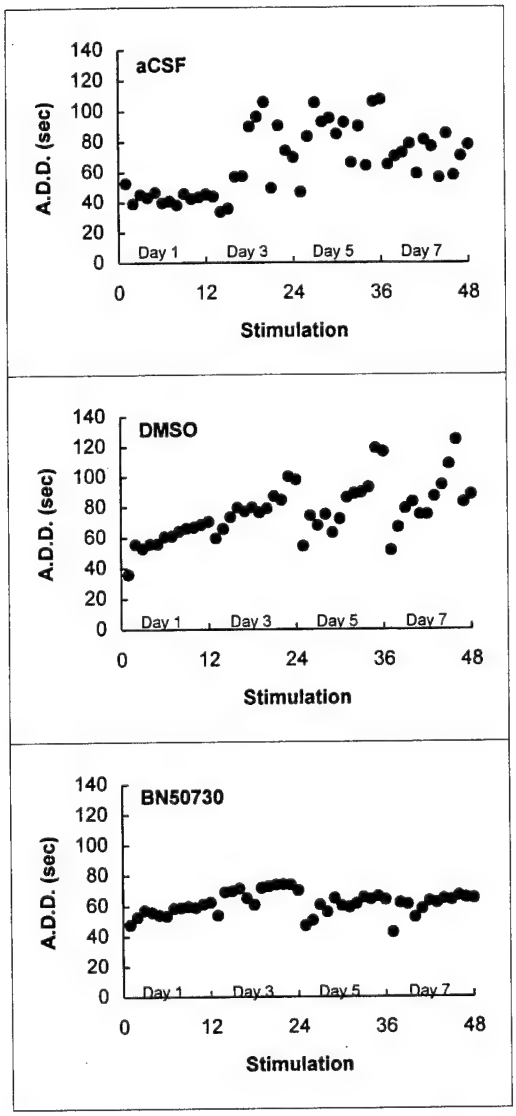


Figure 3. Changes in after discharge duration (A. D. D.) during kindling; effect of PAF antagonist BN50730. Significant differences were observed between treatments (ANOVA, $p<0.0001$)
-No statistical differences were noted during the first day of stimulation between treatment groups.

A-3) The computer vs.manually analyzed ADD is shown in **Figure 4**. By using a computer scope produced by RC Electronics to increase the efficiency and to eliminate any experimental bias during data analysis, we showed that the results obtained by manually analyzing the EEG record are comparable to the results obtained when EEG analysis is done automatically.
-A computer based analyzed record of ADD shows the same trends in lengthening of the after discharge that the record showed when manually analyzed.

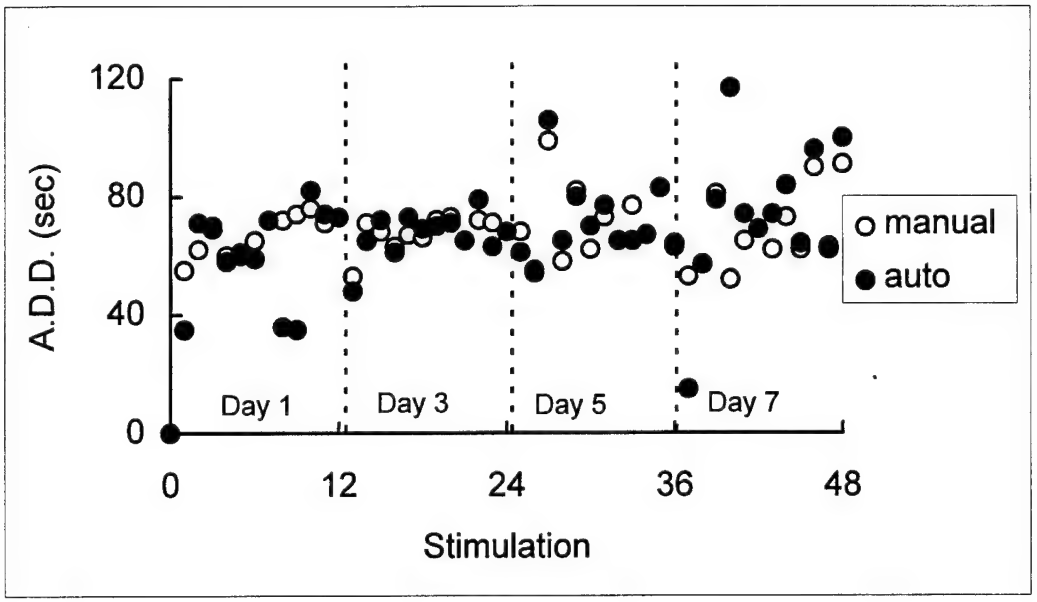


Figure 4. Comparison between manual and automated analysis of A. D. D. Graph depicts the same animal analyzed via computer and manually throughout the whole kindling process (12 stimulations per day every other day for 7 days).

A-4) IEG analysis: work in progress

A-5) Hippocampal histology in kindled rats is shown in **Figures 5A** and **5B.** After kindling every

other day for 7 days, rats were allowed to rest for 4 days. Then animals received twice daily for 15 days kindling stimulation to reach a total of approximately 30 class 5 seizures. Animals were killed 24 hours after the last stimulation, and the hippocampus was dissected and prepared for histology. In kindled rats cresyl violet staining (**Figure 5A**) showed cell loss in the hilus, and TIMM staining indicated mossy fiber sprouting (**Figure 5B**). Moreover, the PAF antagonist BN50730 appears to have a protective effect on cell loss.

CELL LOSS FOLLOWING SEIZURES (CRESYL VIOLET)

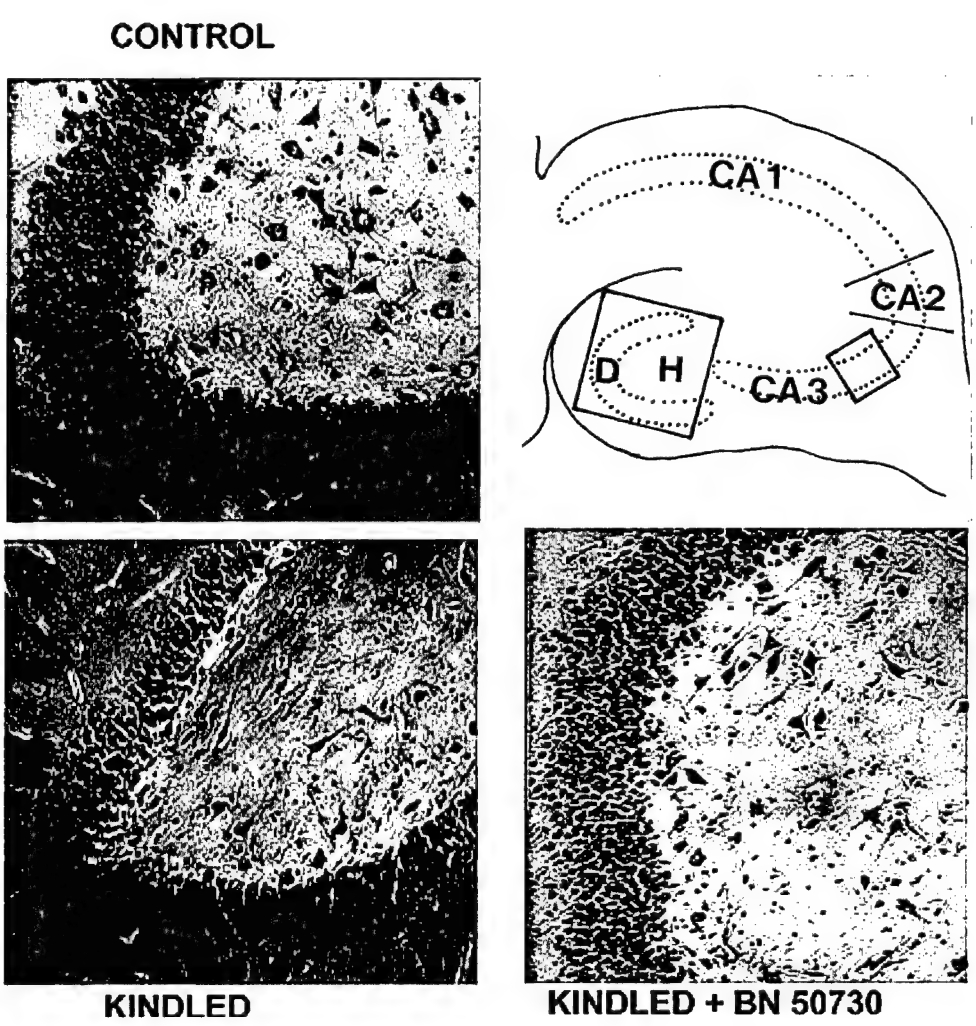


Figure 5A. Hippocampal cell loss in the hilar region of the dentate gyrus during rapid kindling. *Top left* : control. *Top right*: Schematic drawing to illustrate regions within the hippocampus and dentate gyrus from which photographs were taken. Cell-specific regions of hippocampus; CA1, CA2, CA3. D, dentate gyrus. H, hilus. Boxes denote pictured regions. *Bottom left*: 15 days of post kindling stimulation to reach class V seizures. *Bottom right*: as in bottom left, treated with BN50730, demonstrates only moderate cell loss as compared with untrated rats. Cresyl violet stain, 10X.

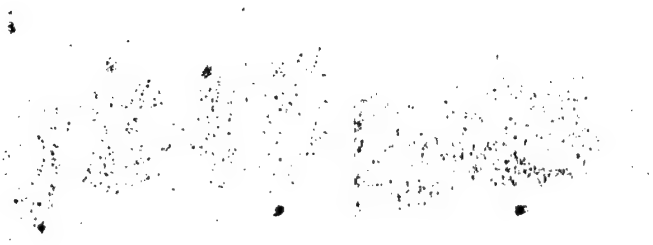


Figure 5B. TIMM-labeled Mossy terminals within hippocampal CA3 region of thin plastic sections. *Left:* control showing mossy fiber terminals in the CA3 region. *Right*: after 15 days of post kindling stimulation to reach class V seizures, showing an increased number of Mossy fibers terminals in the CA3 region (2 visual fields from hylus). The density of terminals is 69% above that of control. Nomarski DIC Optics, 20X.

B-1) ECS triggered:

- a transient increase in TIS-8 (*zif*-268, egr-1) mRNA levels that peaks by one hour and recovers basal values by 2 hours (**Figure 6**).

- a sustained increase in TIS-10 (COX-2) mRNA levels that peaks by 2 hours and slowly returns to basal levels, with values still over control by 24 hours (**Figure** 7).

-70% inhibition of induced TIS 10 (**Figure 9**)

Pretreatment with dexamethasone resulted in:

- 25% inhibition of TIS-8 (**Figure 8**)

- 40% inhibition of TIS 10 (**Figure 9**)

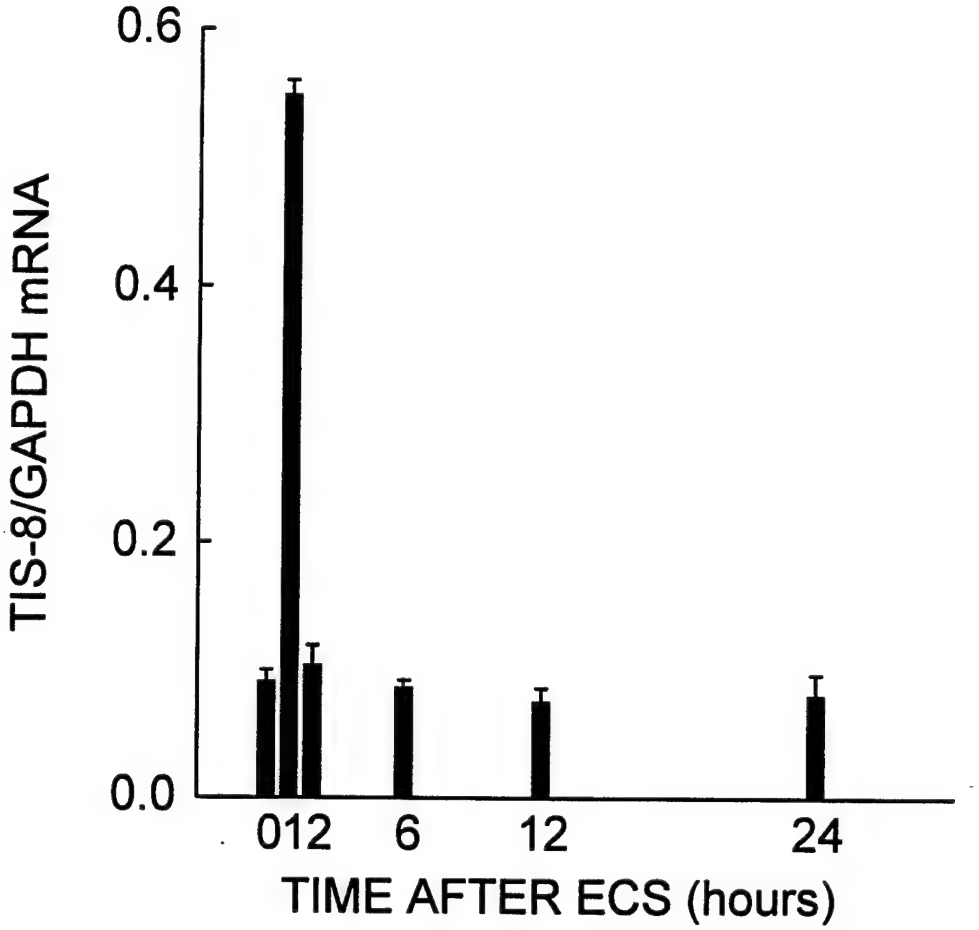


Figure 6. Brain expression of **TIS-8 mRNA** after electroconvulsive shock. Rats were killed at different times after 1-ECS, hippocampus was dissected, mRNA extracted and Northern Blot analysis performed. Blots were hybridized for TIS-8 mRNA. The gene was screened in conjunction with GAPDH as housekeeping gene. Data obtained by phosphor-image quantification was normalized with GAPDH mRNA. Each time point is the average ± SD from at least three individual determinations.

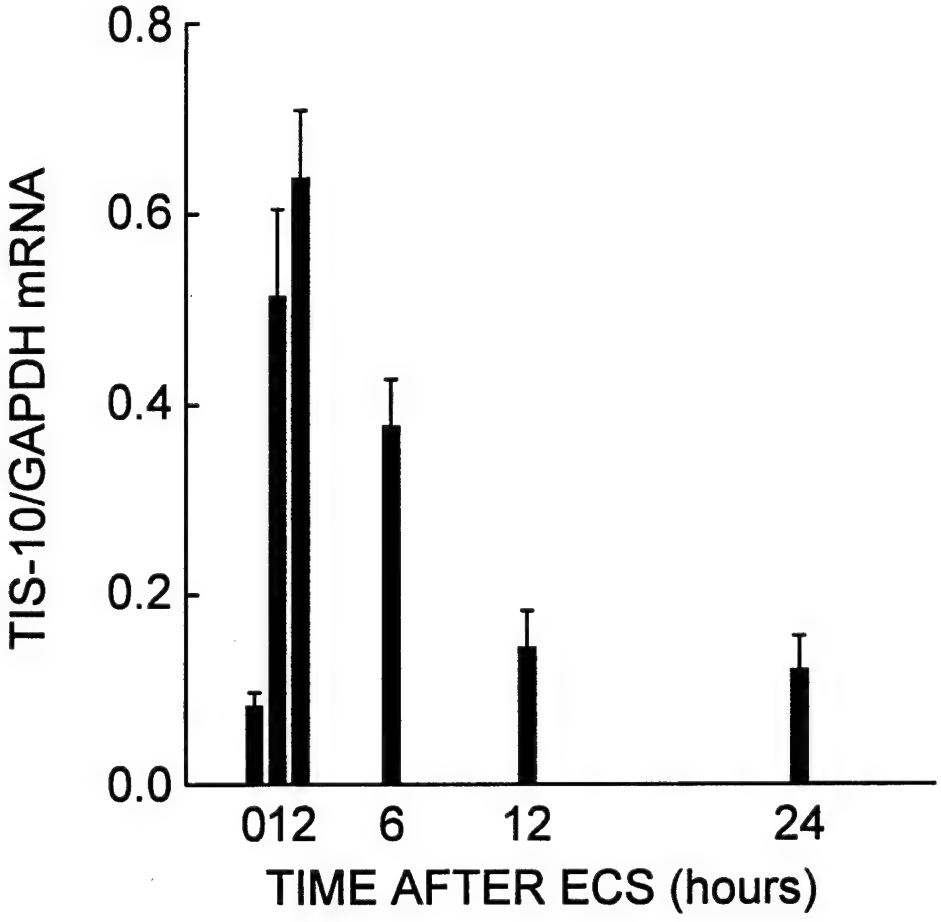


Figure 7. Time course of **TIS-10 mRNA** levels in rat hippocampus after a single electroconvulsive shock. Norther Blots were hybridized for TIS-10 mRNA. Other details as in Fig. 7 legend. **Pretreatment with the PAF antagonist BN50730 resulted in** - 58% inhibition of induced TIS-8 (**Figure 8**)

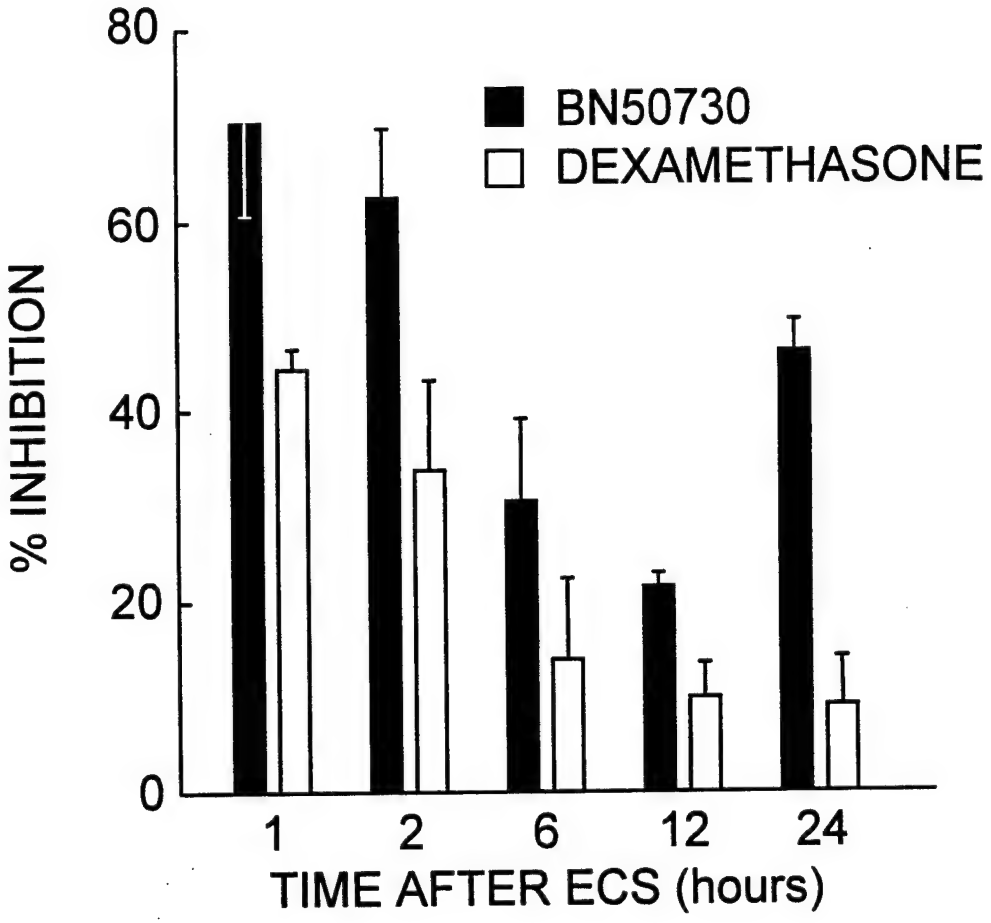


Figure 8. Inhibitory effect of BN50730 and dexamethasone on ECS-induced **TIS-8 mRNA** expression in rat hippocampus. BN50730 or vehicle were *icv* injected 30 min before ECS. Dexamethasone disolved in DMSO:saline (2:1, v/v) was administered *ip* 6.2 mg/Kg body weight each 8 hours, starting 24 hours before ECS.Other details as in Fig. 7.

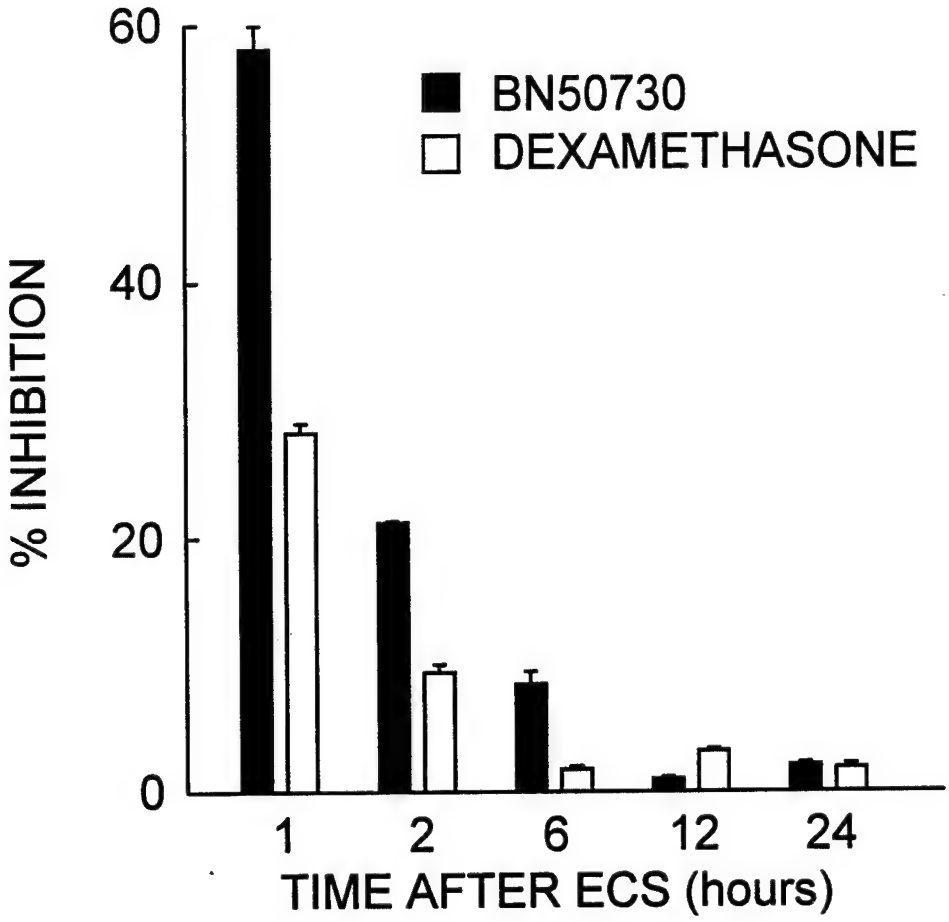


Figure 9. Inhibitory effect of BN50730 and dexamethasone on ECS-induced **TIS-10 mRNA** expression in rat hippocampus. Details as in Figs. 8-9

B-2) *In vitro* transcription assay corroborates:

- that ECS triggers a fast activation of TIS-10 gene expression that peaks by 2 hours and an

earlier and smaller effect in mRNA TIS-8 levels (**Figure 10**).

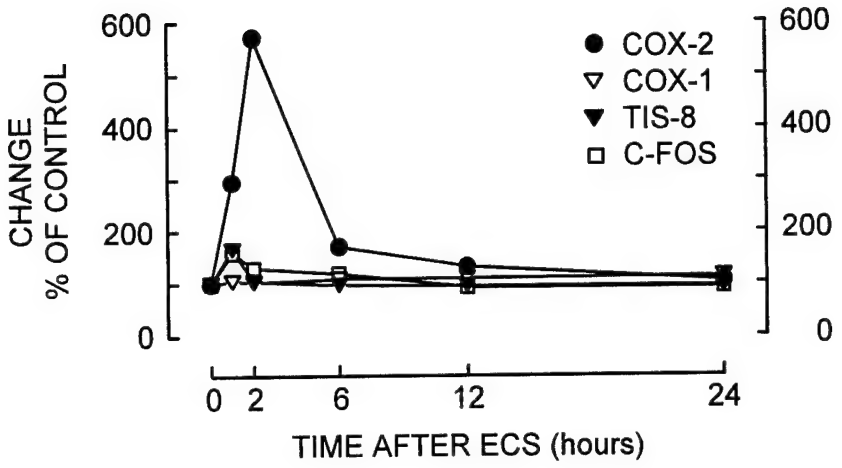


Figure 10. Activation of *in vitro* transcpription after a single ECS in the rat hippocampus. Nuclear samples for each time point were prepared, and figures are the combined results of two experiments.

7- SUMMARY AND CONCLUSIONS

A- The infusion of the PAF antagonist BN50730 during kindling produced a repression of the lengthening of ADD normally associated with the kindling phenomenon. The data showed statistically significant differences between treatment groups using an ANOVA test. In addition, differences were seen between treatment groups with respect to behavioral seizure scores. Not only did the BN50730 animals experience less intense seizures, but the progression of seizure activity increased at a slower rate than it did in the DMSO-treated animals.

B- Northern Blot and Nuclear run-off studies revealed that ECS activates IEG expression in the hippocampus and that PAF mediates these effects.

- **TIS-10 (COX-2) mRNA**: showed a sustained upregulation which was inhibited by BN50730. This may imply ECS ⇨ PAF ⇨ receptor ⇨ TIS-10 mRNA ⇨ PGHS-2 (inducible prostaglandin synthase) ⇨ prostaglandins.

- **TIS-8 (*zif*-268) mRNA**: showed a transient increase, inhibited by BN50730. This may imply ECS ⇨ PAF ⇨ receptor ⇨ TIS-8 (transcription factor) ⇨ gene cascade (synapsin 1) ⇨ modulation of neurotransmitter release.

RESEARCH PLAN FOR THE UPCOMING YEAR

- Further EEG analysis during kindling in control and BN50730-treated rats.
- IEG expression during kindling (TIS-8, TIS-10). Work is currently in progress.
- Study the response of hippocampal cells to kindling at the level of the expression of rapidly responding transcription factors, DNA binding proteins that appear to be involved in the expression of injury response genes. To this end cell nuclei from control rats and experimental ones killed at different times during kindling will be subject to electrophoretic mobility shift assay (EMSA) following the protocol of Fried and Crothes (1981) as modified by Lukiw et al., (1994).
- Continue with histological analysis of hippocampal tissue during kindling and the involvement of PAF in mossy fiber sprouting and cell death. This work is currently in progress.
- Study the expression of mRNA GAP-43, associated with axonal sprouting and growth cone guidance (Coggins and Zwiers, 1991), in cortex and hippocampus using two experimental models: the rapid hippocampal kindling model and ECS.

ABSTRACTS AND PUBLICATIONS

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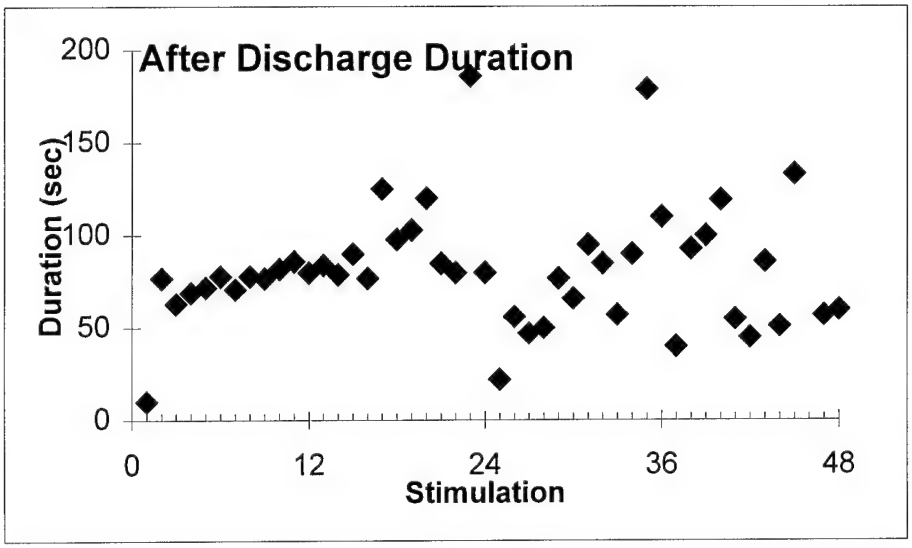
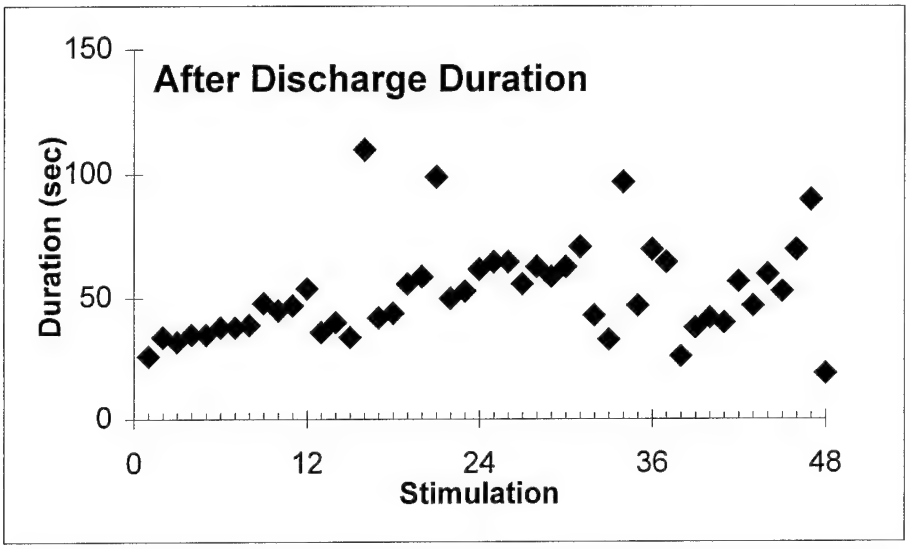
Appendix:

The following 19 pages are the individual records during the kindling process of After Discharge Duration and Behavioural Seizure Scores for Sham animals (DMSO treated, n=7); Sham (CSF, n=2); and BN50730 treated (n=10).

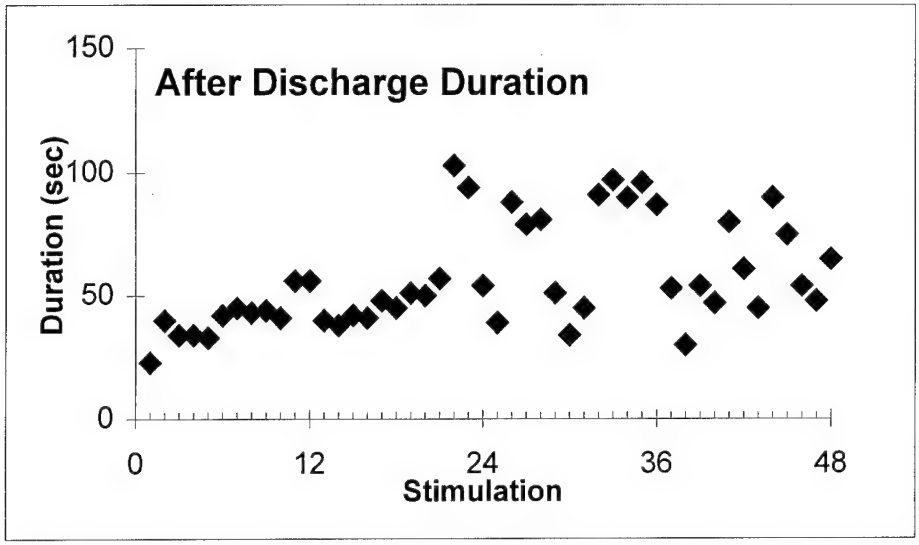
Rat 4 (exp. 1): Sham DMSO treated



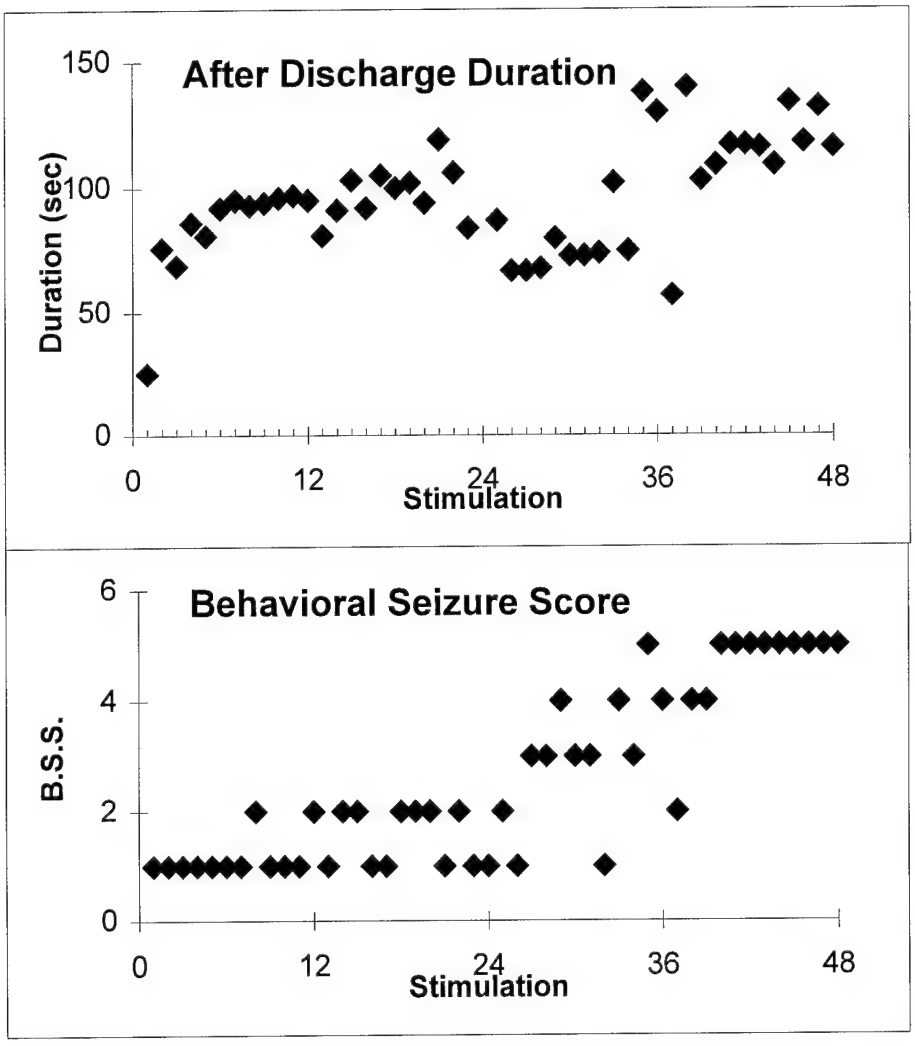
Rat # 5 (exp. 1): Sham DMSO treated



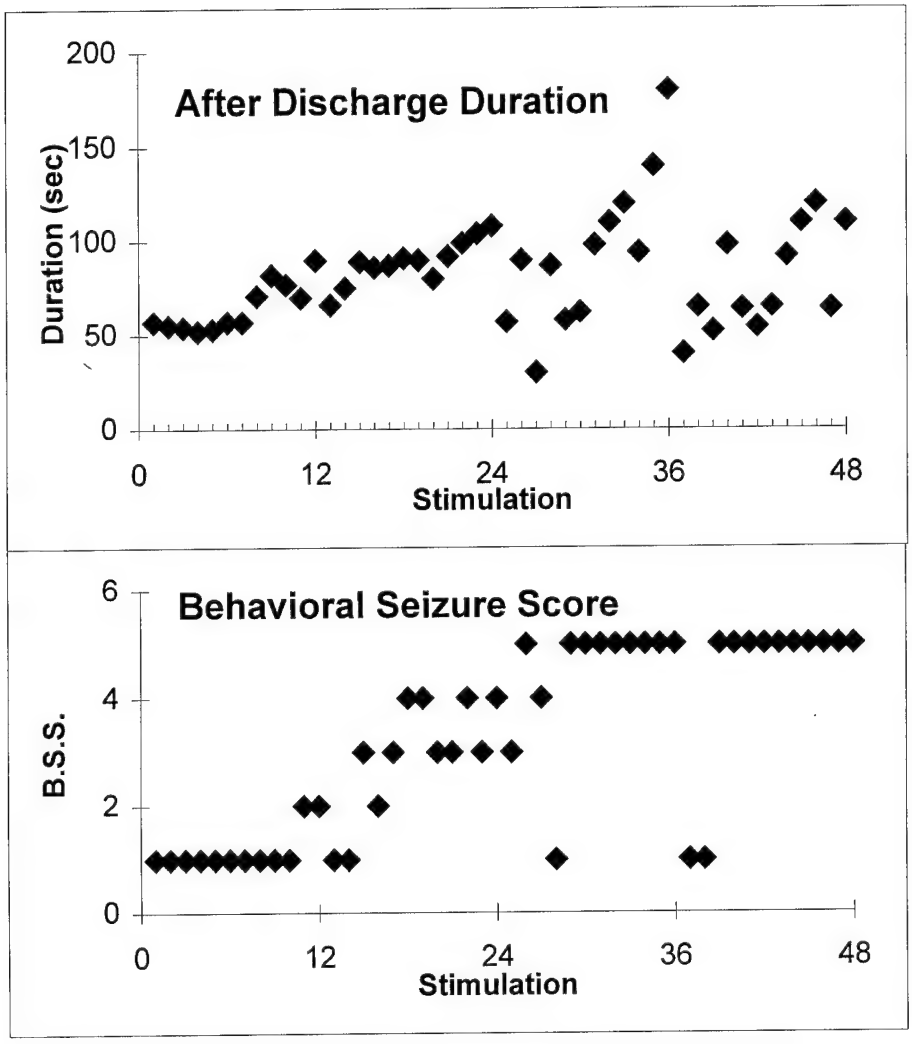
Rat # 6 (exp. 1): Sham DMSO treated



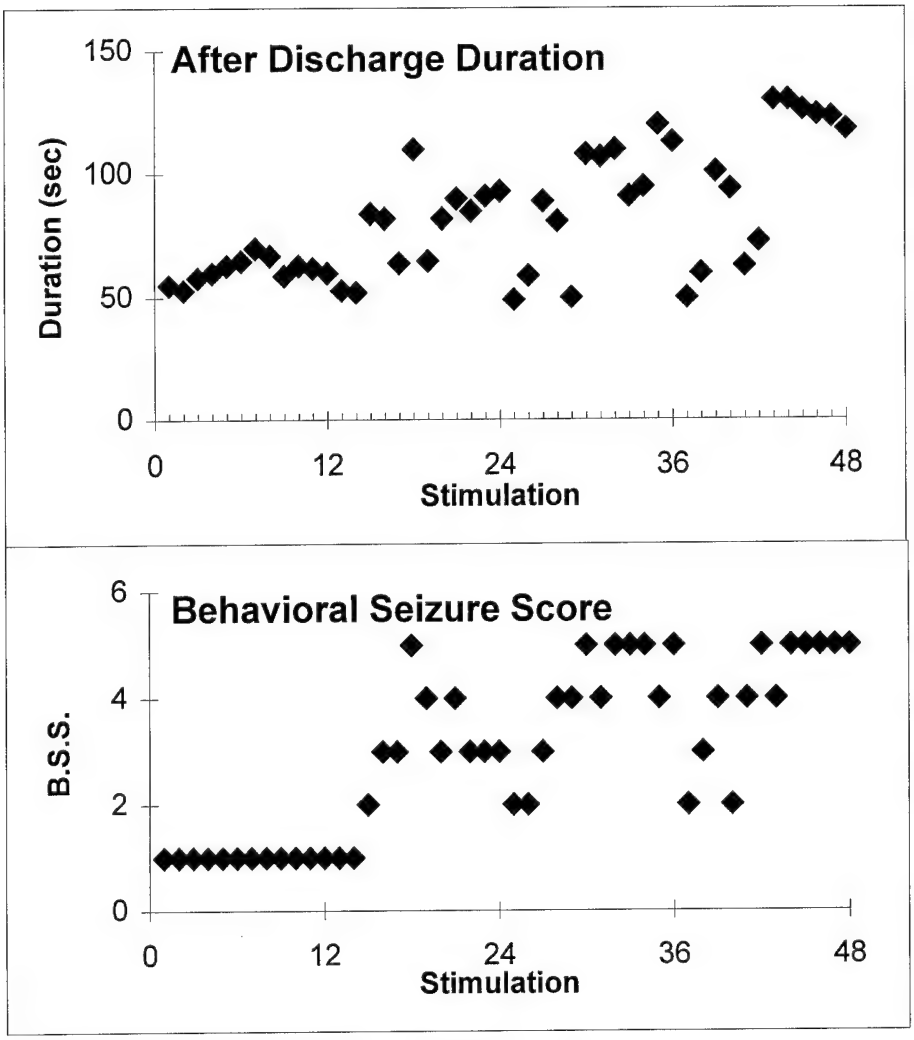
Rat # 8: Sham DMSO treated



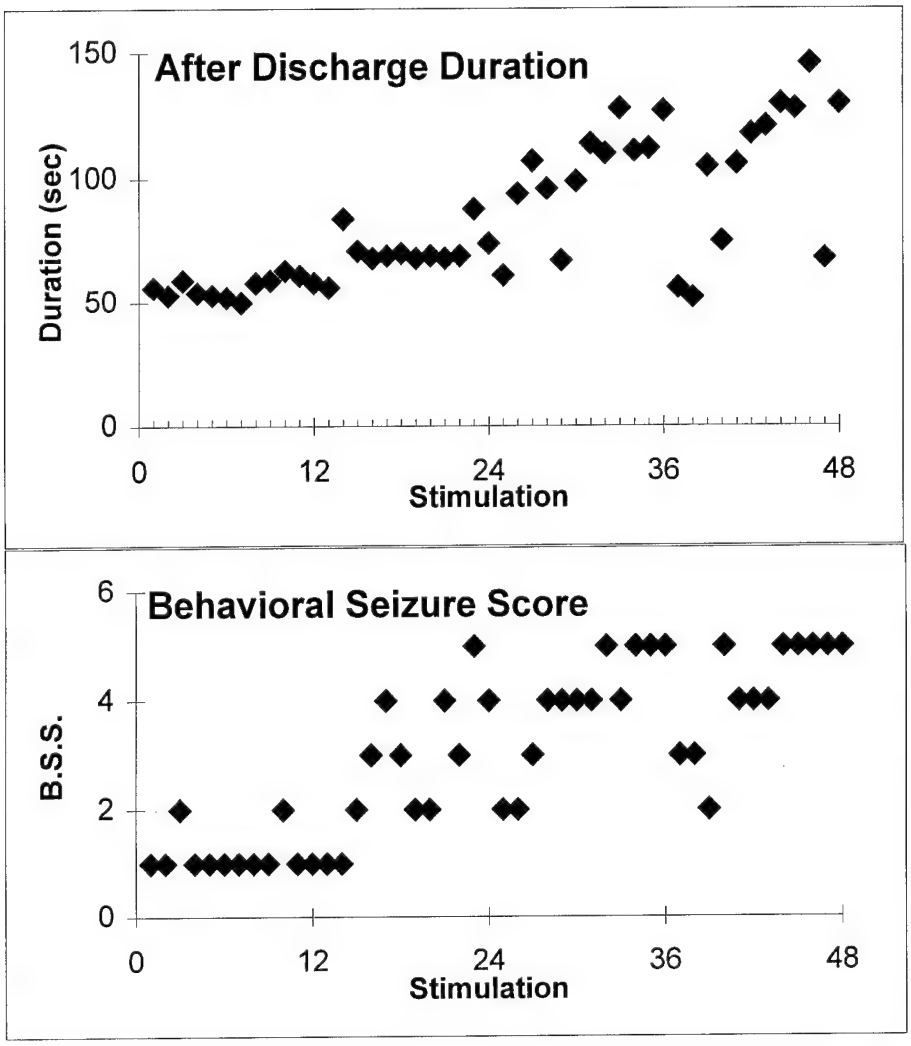
Rat # 10: Sham DMSO treated



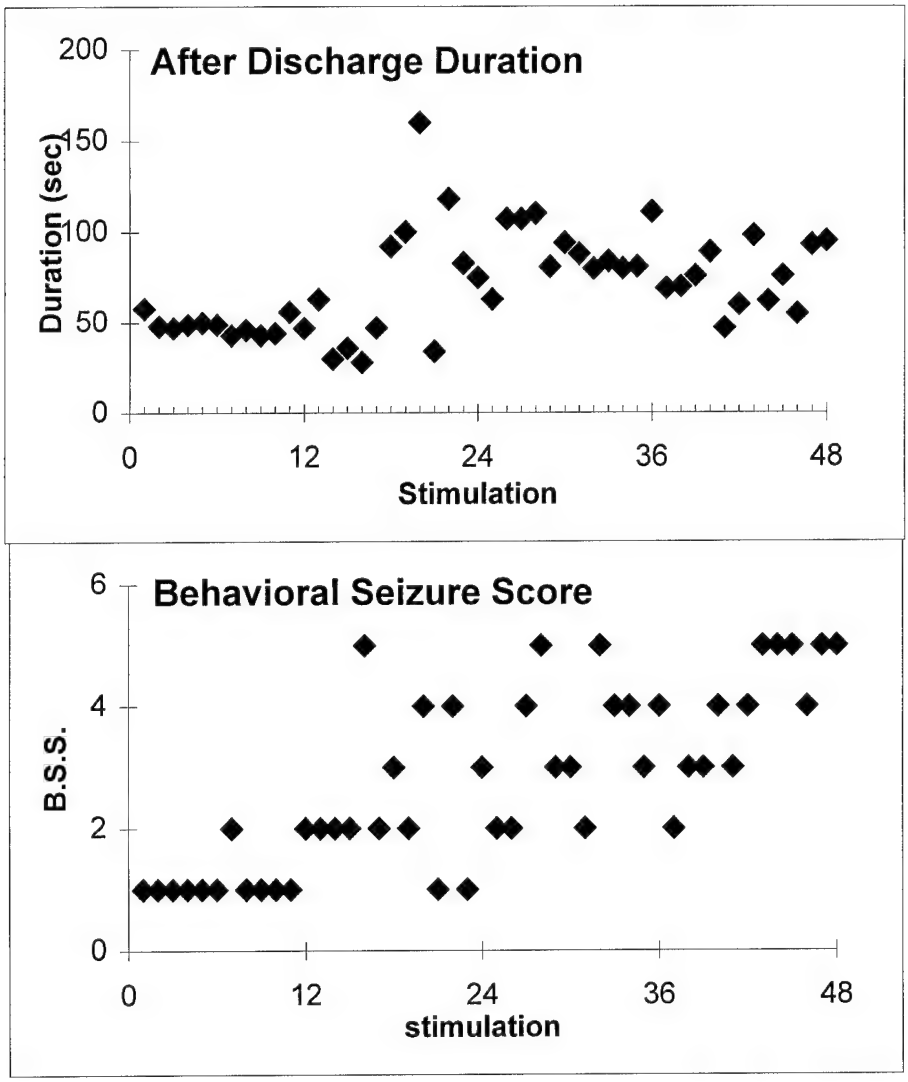
Rat # 11: Sham DMSO treated



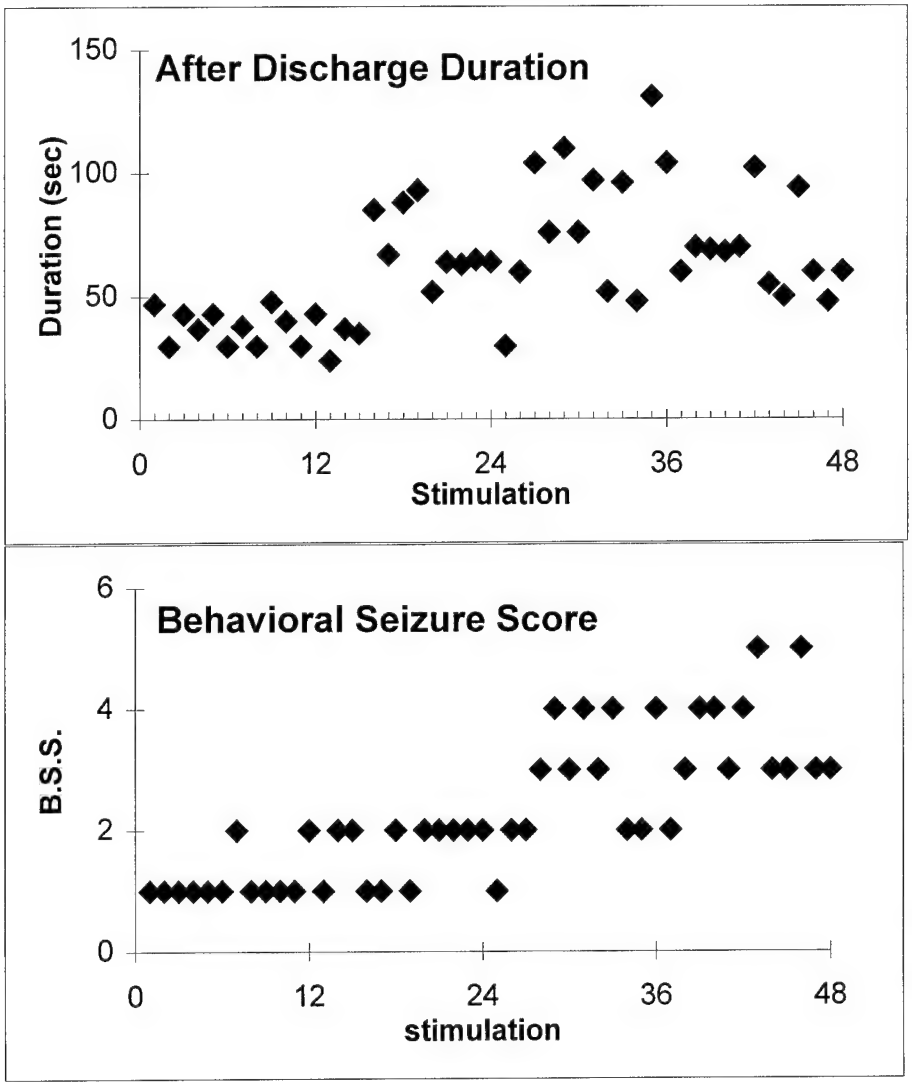
Rat # 14: Sham DMSO treated



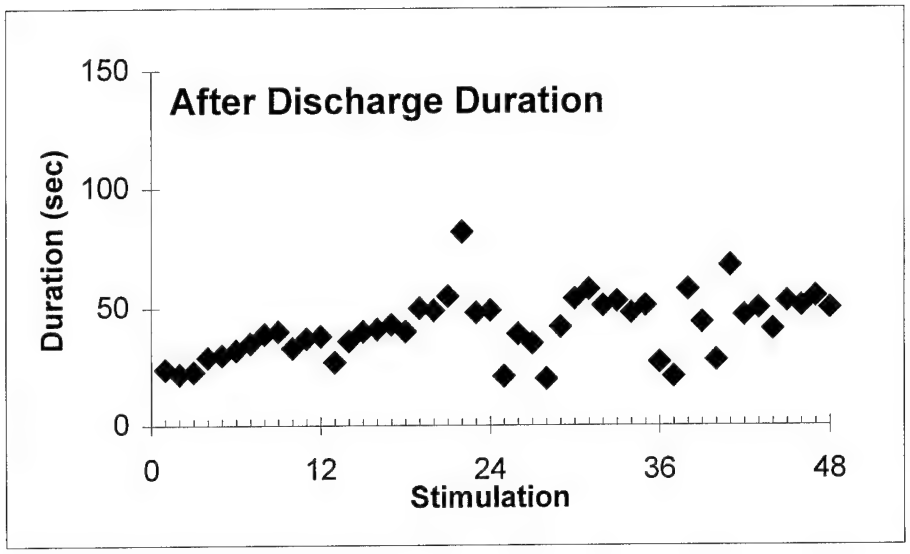
Rat # 31: aCSF treated



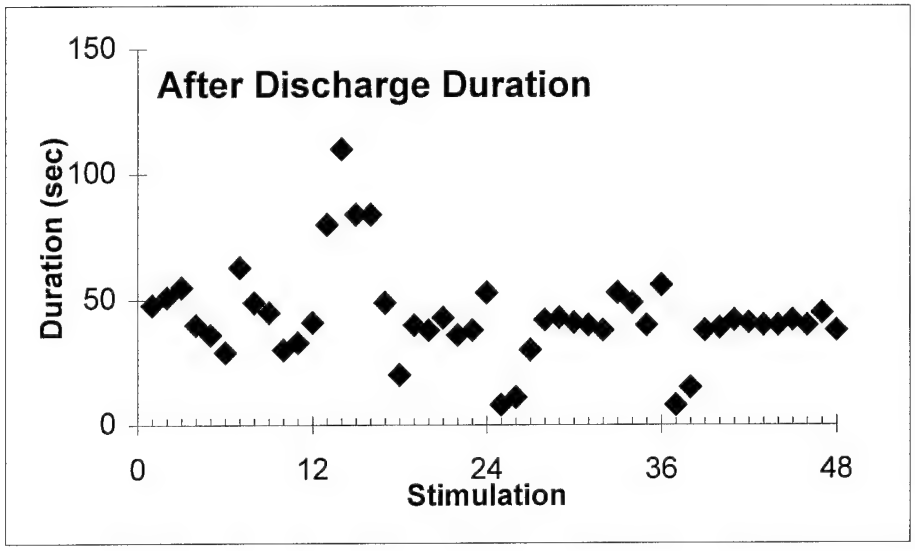
Rat # 32: aCSF treated



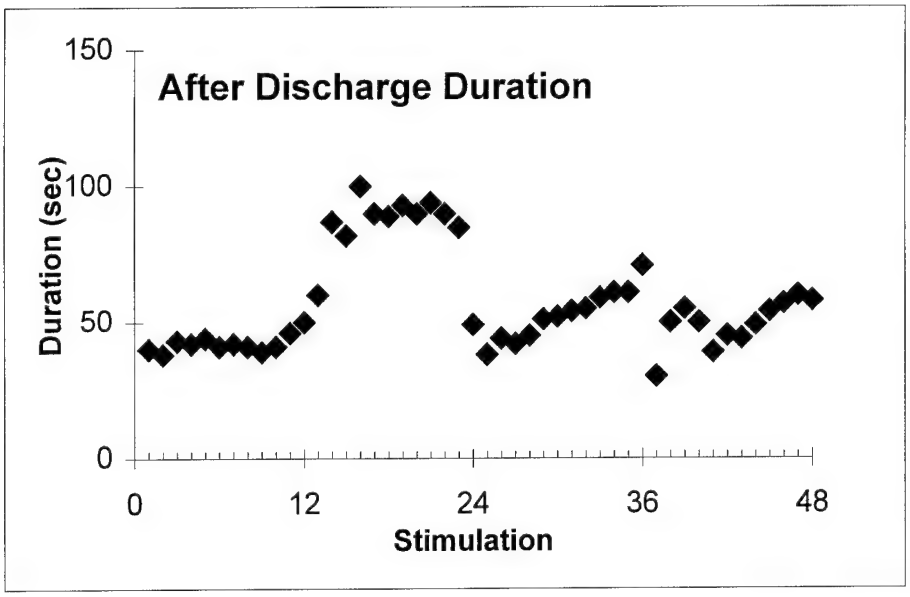
Rat # 1 (exp 1): BN50730 treated



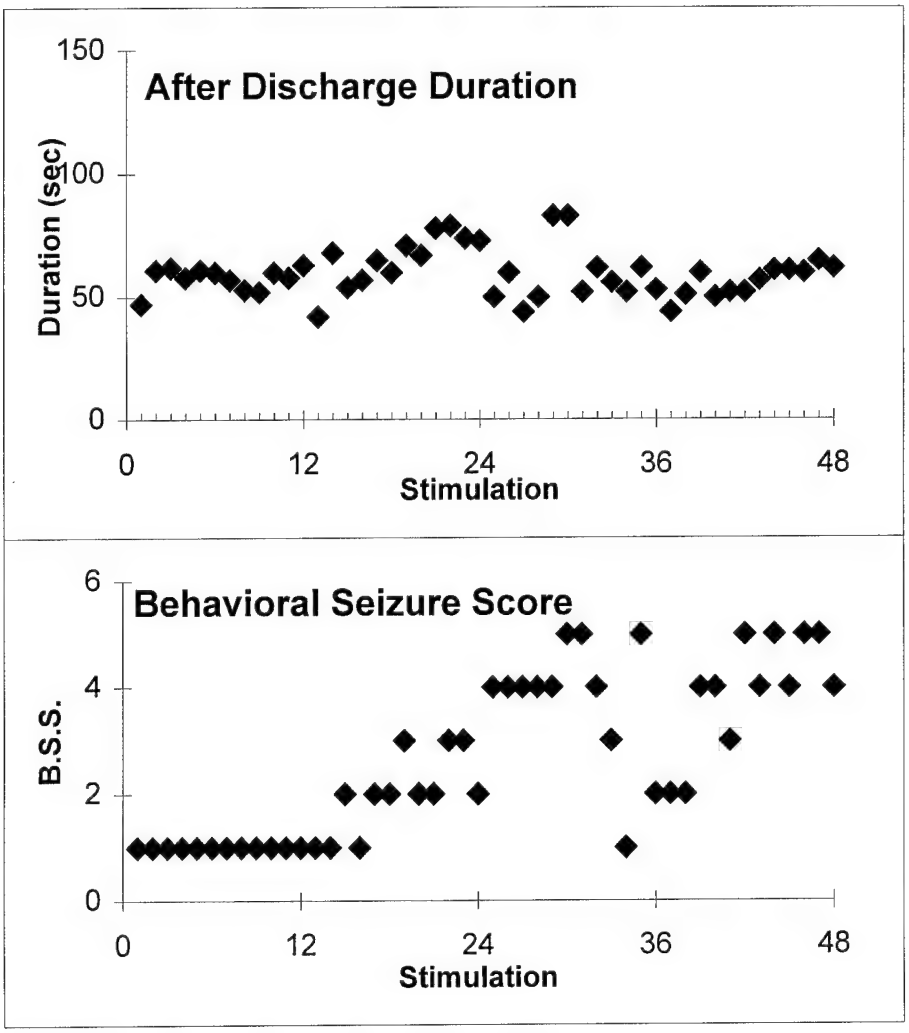
Rat # 2 (exp. 1): BN50730 treated



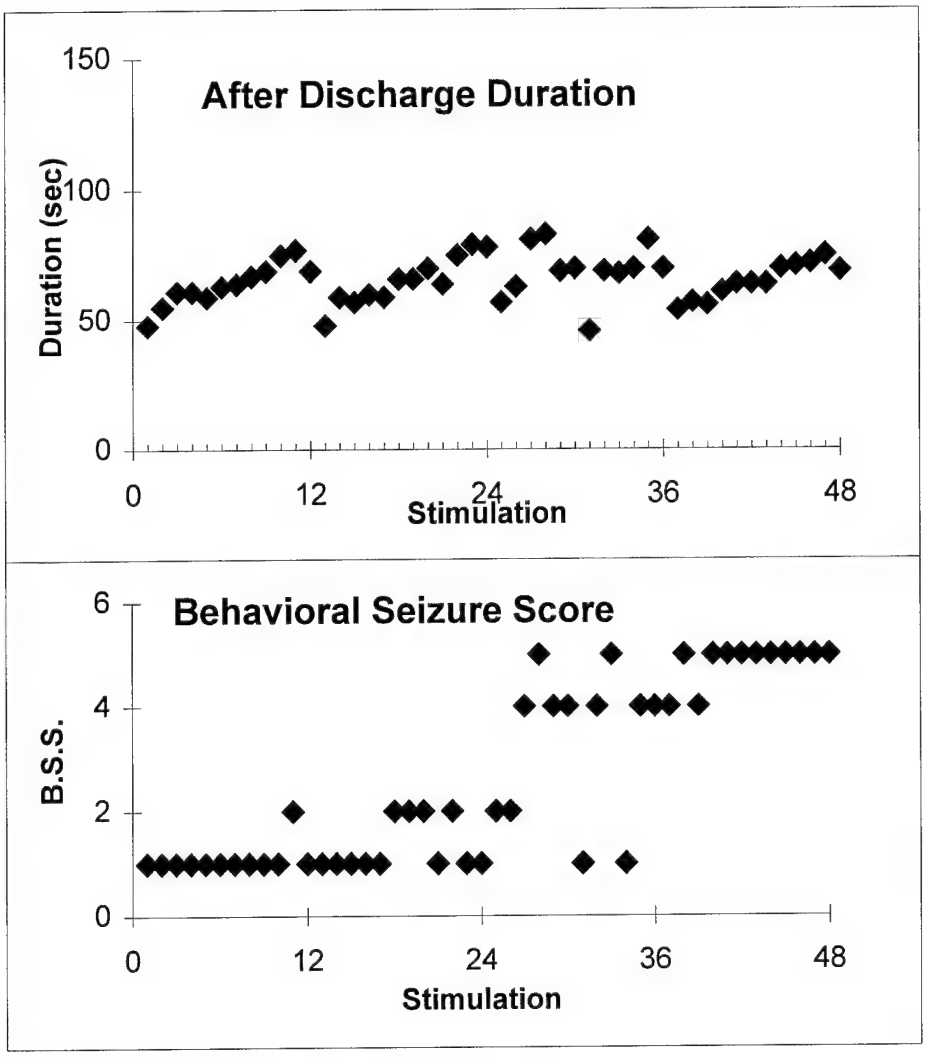
Rat # 3 (exp. 1): BN50730 treated



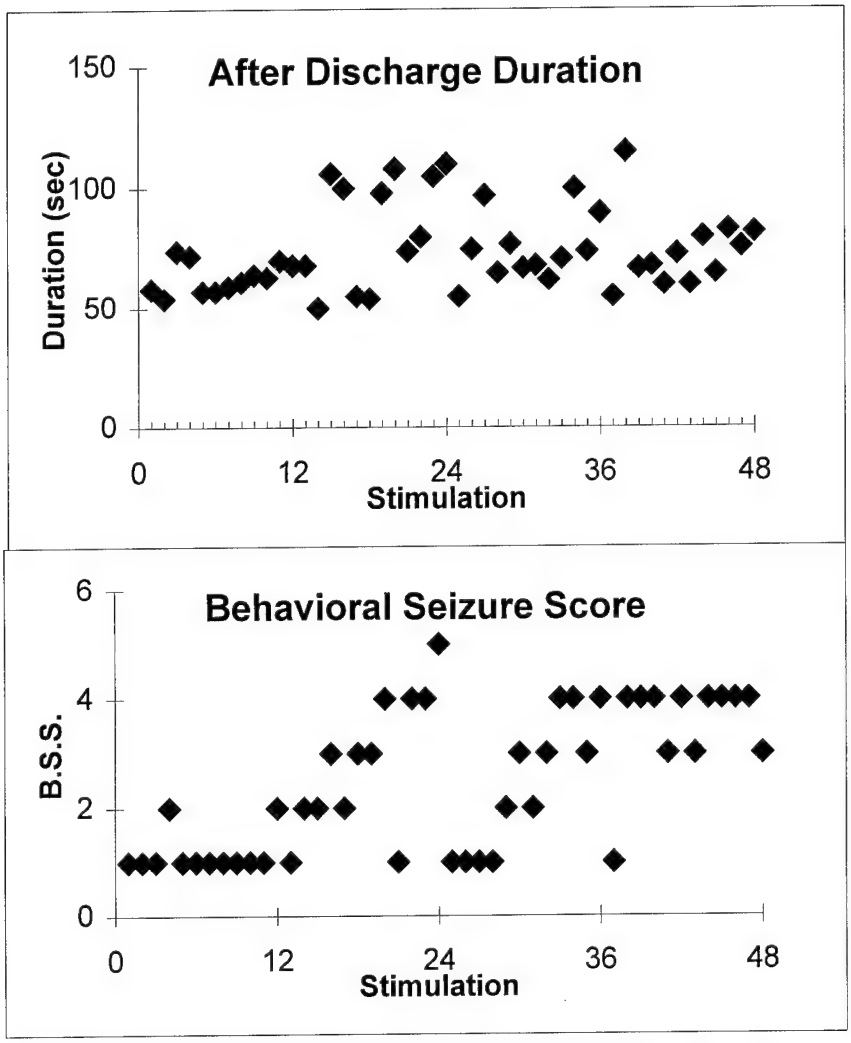
Rat # 1 (exp, 3): BN50730 treated



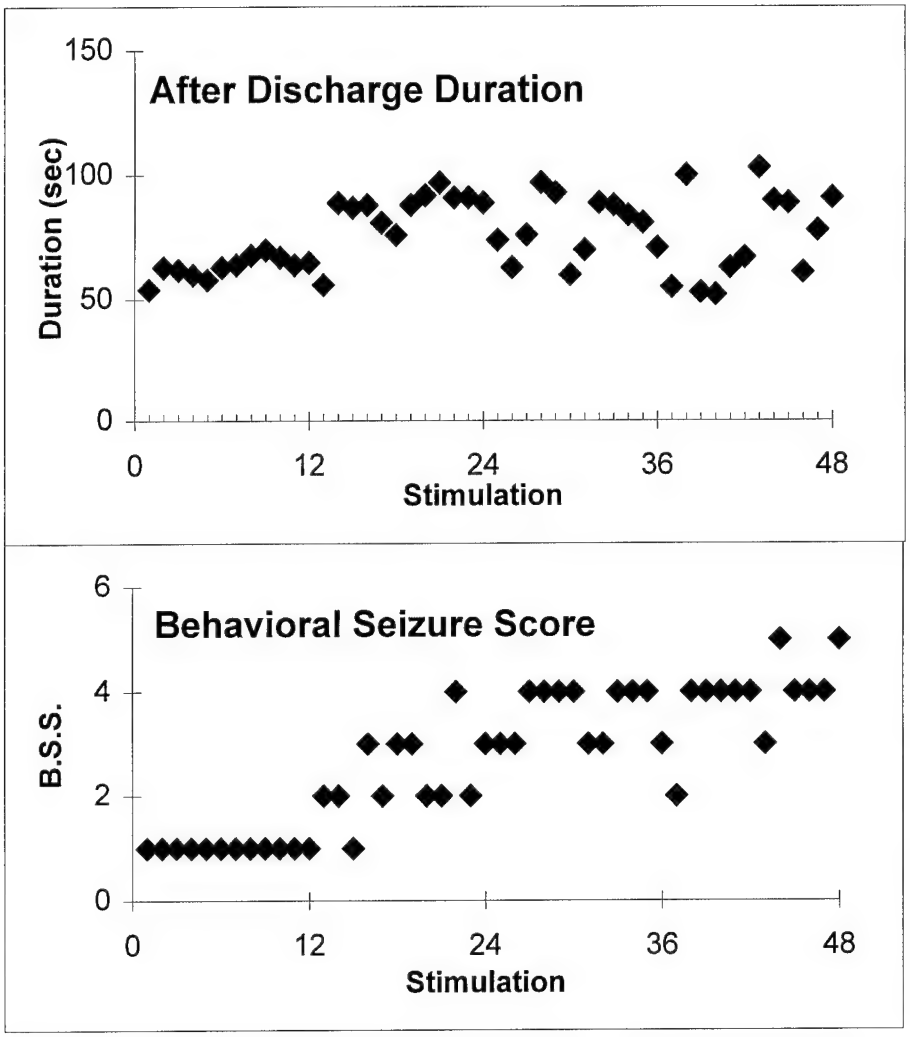
Rat # 3: BN50730 treated (Exp. 2)



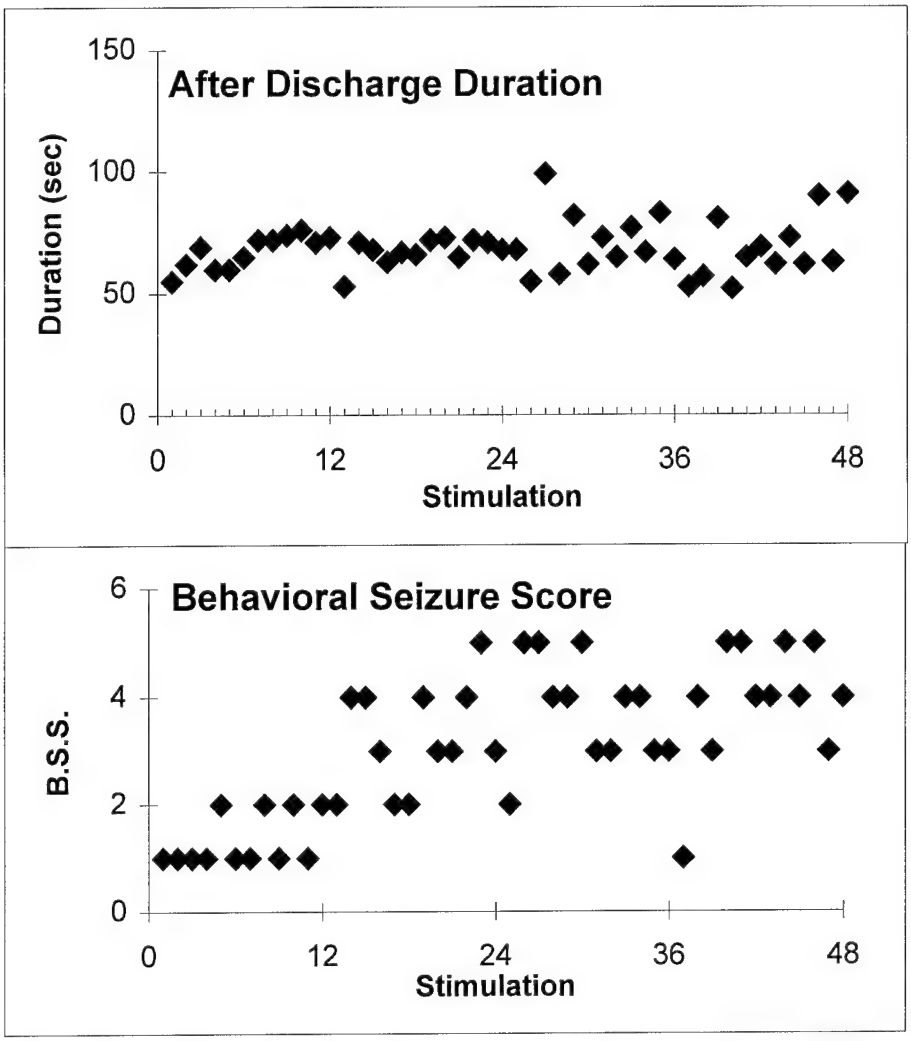
Rat # 5: BN50730 treated



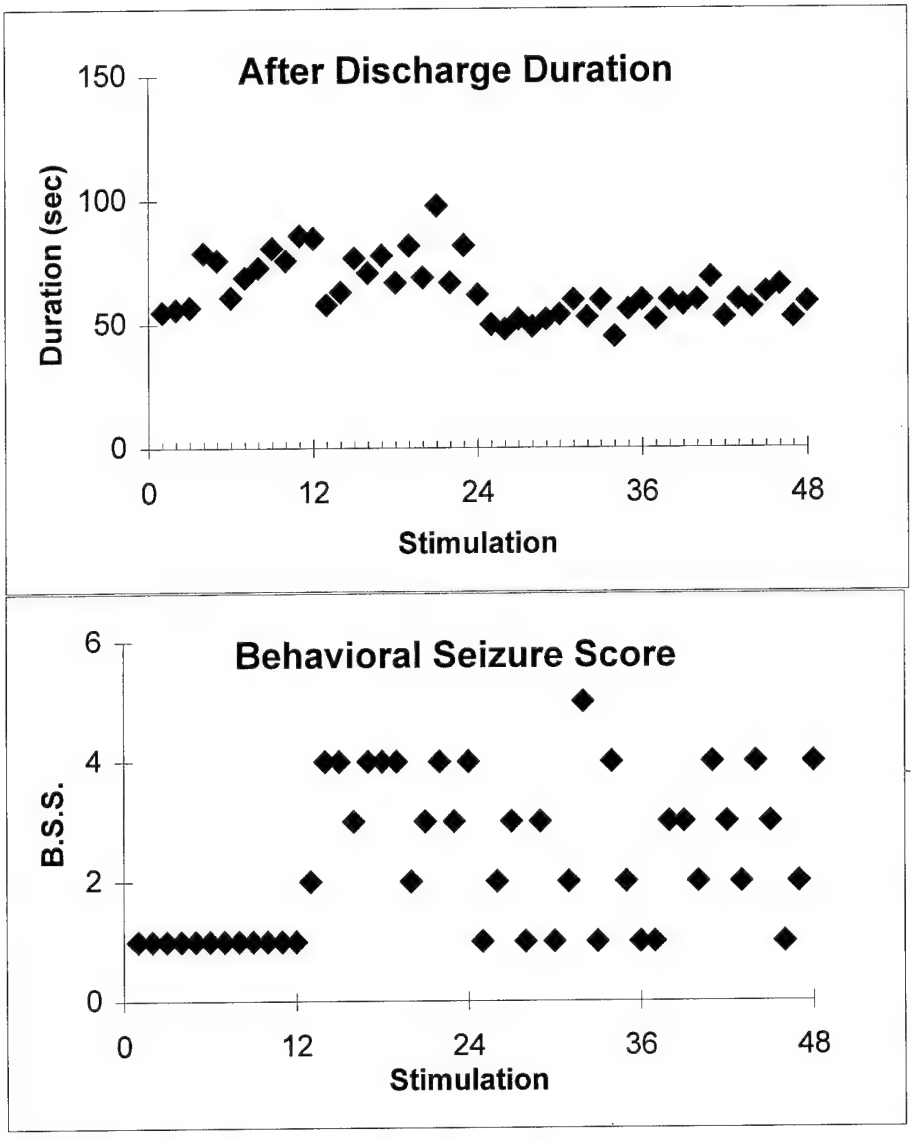
Rat # 6 (exp. 3): BN50730 treated



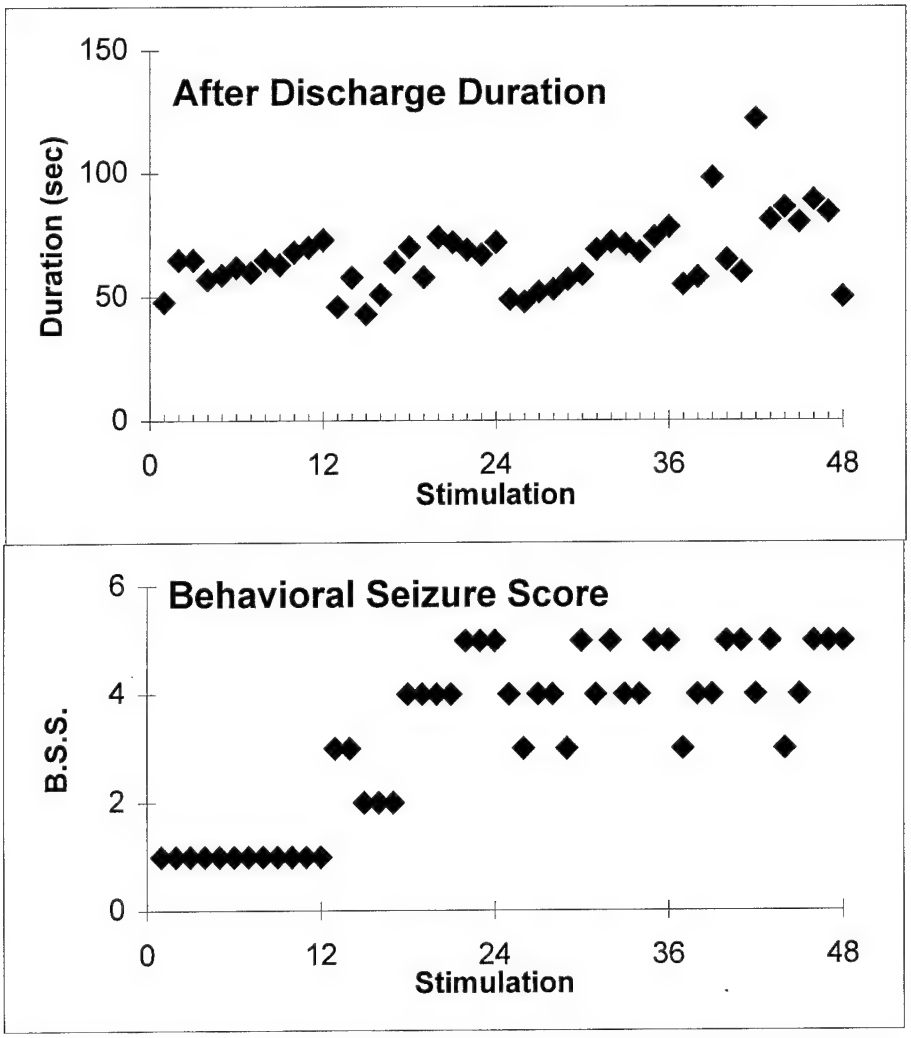
Rat # 7 (exp. 3): BN50730 treated



Rat # 9: BN50730 treated



Rat #18 : BN50730 treated



TITLE: NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR.

CHAPTER: NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR.

TRAUMATIC BRAIN INJURY

TABLE OF CONTENTS

INTRODUCTION

Exploration of the molecular pathophysiology of neurotrauma is providing new clues for the development of novel and more effective therapeutic targets to limit neuronal damage, slow cell death, and promote repair of the damaged tissues. At the onset of traumatic brain injury (TBI), a cascade of excitotoxic and neurotoxic events is activated, which leads to increased neuronal calcium levels and the accumulation of injury mediators (Faden et al., 1989; Fineman et al., 1990). These mediators may activate gene cascades that, in turn, initiate either neuronal cell damage and death or repair-regenerative responses (Bazan et al., 1993; Bazan, 1994). TBI activates complex biochemical events which lead to long-term neurobehavioral deficits (Hayes et al., 1992).

As a consequence of TBI, ischemia , and seizures, neurotransmitters are released and, in turn, generate an overproduction of second messengers. A key trigger player of excitotoxic neuronal damage is the excitatory amino acid glutamate (Olney, 1986; Rothman and Olney, 1986), which is released and accumulates in the brain after TBI (Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990) and ischemia (Benveniste et al., 1984; Meldrum, 1990; Mitani et al., 1990; Christensen et al., 1991). Glutamate triggers increased permeation of calcium mediated by NMDA receptors, and activation of phospholipase A_2 (PLA_2) in postsynaptic neurons (Rothman and Olney, 1986; Choi, 1988; Meldrum and Garthwaite, 1990). Activation of PLA_2 (Shojami et al., 1989) and release of arachidonic acid (AA) (Dhillon et al., 1994) are among the lipid-derived membrane signaling systems activated after TBI. Free AA may directly affect neuronal synaptic activity (Volterra et al., 1992) or can be further metabolized to prostaglandins and leukotrienes (Shimizu and Wolfe, 1990). These metabolites are capable of not only modulating synaptic transmission (Piomelli, 1994), but also of affecting the microvasculature prostaglandins by affecting cerebral blood flow (Moncada and Vane, 1979) and leukotrienes by increasing the permeability of the blood-brain barrier (Unterberg et al., 1987; Babna et al., 1991). In fact, increased production of prostaglandins (Ellis et al., 1981; Dewitt et al., 1988; Shojami et al, 1987) and vasogenic brain edema (Tanno et al., 1992; Soares et al., 1993) have been observed after TBI.

Activation of PLA_2 can stimulate the synthesis of platelet-activating factor (PAF), a mediator of inflammatory and immune responses (Braquet et al., 1987) that has also been linked

to ischemia/reperfusion-induced brain damage (Panetta et al., 1987). PAF actions are mediated through its interaction with receptors found extracellularly in presynaptic membranes and intracellularly in microsomal membranes (Marcheselli and Bazan, 1990; Bazan et al., 1991). There are several PAF antagonists which block these receptors. The bioactivity of PAF towards the presynaptic receptors is to stimulate glutamate release; the antagonist BN52021 blocks this effect (Clark et al., 1992). PAF activates early genes transcription (*c-fos, c-jun, zif-268*) by interacting with intracellular binding sites, and this effect is blocked by another PAF antagonist: BN50730 (Squinto et al., 1989; Bazan et al., 1991). Similarly, in the hippocampus, BN50730 blocks the ECS-induced activation of c-*fos* and *zif*-268 genes (Marcheselli and Bazan, 1994)

COX-2 (Tis 10, PGS-2, inducible cyclooxygenase), a recently cloned gene, is also a member of the immediate-early gene family (DeWitt et al, 1988; Kujubu and Herschman, 1991). COX-2 encodes one subtype of the enzyme Prostaglandin Synthase-2 (PGHS-2), and is rapidly induced in the neural tissues by a different sort of stimuli, which includes PAF (Bazan et al., 1994). In contrast, Prostaglandin Synthase-1 or PGHS-1, (also called Cox-1,) is a constitutively expressed enzyme (DeWitt and Smith, 1988.). Even though both proteins have 90% amino acid sequence homology, they display significant differences in pharmacological effects (Herschman, H. R. 1994). COX-2 and the constitutively expressed PGHS-1 may contribute to the production of eicosanoid from free AA (Hershman, 1994) the latter under normal basal conditions and the former when synaptic activity is overstimulated (i. e. after ischemia, TBI, seizures).

Two models of brain injury have been set up in our laboratory and are described in previous reports: a percussion device that generates hydraulic impacts of selected pressures, and a cryogenic injury model that generates vasogenic edema. As mentioned above, edema formation, as a consequence of blood-brain barrier breakdown, has been observed after TBI. These experimental approaches allow us to continue our study into the temporal sequences of neurochemical pathways activated after neurotrauma which lead to delayed neuronal death and/or recovery, the involvement of PAF as an activator of gene cascades in the pathophysiology of TBI, and the testing of new neuroprotective drugs.

BODY.

Previous work.

In the previous report (**year 1**), mRNA expression of *Zif*-268 and Tis-10 (Cox-2, Cyclooxygenase-2, or PGHS-2) genes in cryogenically injured rats was shown. We reported that transcription of the COX-2 gene, to a greater extent than that of *Zif*-268, is activated by injury and is sustained up to 24 hours post injury. Moreover, pretreatment with the PAF antagonist BN50730, selective for intracellular PAF binding sites (Marcheselli et al., 1990), inhibits increased levels of mRNA more effectively than dexamethasone. At present, a publication is being prepared which includes the first year of the study.

A rapid and sensitive fluorometric method to detect brain edema using a Evans Blue extravassation technique was also validated during the first year. This experimental model was used during the second year to test the ability of a new PAF antagonist to inhibit injury-mediated vasogenic edema.

Objectives, year 2.

The response of rat neuronal cells to tissue damage induced by cryogenic brain injury was examined in the rat brain cortex and hippocampus at the level of (**A**) the expression of rapidly responding transcription factors, and (**B**) COX-2 protein levels by Western Blot analysis. A new PAF antagonist, the LAU-503 compound, was tested for its ability to protect the brain from cryogenic brain injury-induced edema and the expression of early genes TIS-8/TIS-10.

Brain cell nuclei from cryogenically injured rats and control animals were isolated briefly, following a time course after injury, and were assayed for their content of rapidly responding transcription factors (DNA binding proteins that appear to be involved in the expression of injury response genes.) These transcription factors can be classified into two general types: those which reside in the cytoplasm of cells in a latent form, and those which must be generated *de novo* by transcriptional activation of their respective genes. The RNA messages of these latter genes are translated and mobilized into the nucleus where they activate their target genes. Characteristics of the *latent* and *de novo* generated transcription factors, as well as their DNA binding consensus sequences, are outlined in Tables 1a and 1b.

The inducible cyclooxygenase 2 (COX-2, Tis-10) gene is a primary genetic response element that codes for the inducible form of prostaglandin synthetase PGHS-2. This enzyme,

which catalyzes the oxidation of arachidonic acid derived from membrane phospholipids, represents the rate-limiting step in the synthesis of prostaglandins. Prostaglandins are potent mediators of cellular damage induced by brain injury. Protein-DNA binding in the controlling elements, i.e. in the proximal promotor of the inducible cyclooxygenase-2 (COX-2) gene, were examined during the course of cryogenic brain injury. Another aim of the current studies was to determine if the increase in the COX-2 (TIS-10, PGHS-2) mRNA levels induced by cryogenic injury (Previous report, **year 1**) leads to increased levels of the protein PGHS-2. The inducible PGHS-2 was studied by Western Blot analysis. In these studies, animals pretreated with the PAF antagonist BN50730 were compared with vehicle control animals (work in progress).

Methods.

Cryogenic Injury Protocol

The animal model of vasogenic brain injury was generated by the placement of a liquid nitrogen-cooled probe, (b.p. -195.79°C), against the exposed skull of the rat for one minute. The cold probe consisted of a brass rod of 9 mm in diameter and 25 mm in length, with a concave tip that fit against the curved surface of the skull. The probe was attached to a larger brass rod 16mm in diamter and 55 mm in length, which acted as a heat sink and was bound to a 30 cm stainless steel handle. The handle was insulated toward its end with Delrin^{R} to avoid injuries to the operator during the experimental procedures. This probe was immersed in liquid nitrogen until the moment it was applied to the skull surface.

Albino Sprague Dawley rats weighing 150-220 gm were subjected to ether anesthesia, and their skulls were exposed by an incision along the scalp to the midline. The cold probe was then rapidly pressed onto the right fronto-parietal region of the skull for a period of one minute and the animals were sacrificed after injury at time intervals of 0.5, 1, 2, 3, 6, 8, 10, 12, and 24 hours. A control group of 3 animals was alloted. These animals had undergone the same procedure except for probe application. Brain tissues were then rapidly dissected on an ice cold dissection board and the rat brain cortex and hippocampus were rapidly removed.

Evans blue extravasation assay for the detection of brain edema.

One hour prior to injury, animals received a 2 ml *iv* injection of 2 % Evans Blue in saline solution. At different times after injury, animals were killed, and left and right brain cortex were

dissected. A 200 µl blood sample was also collected. Samples were homogenized in 1 ml of 50 % trichloroacetic acid, followed by centrifugation at 10,000 rpm for 20 min at 4°C in a JA-7.5 rotor (Beckman®). Samples of 1 ml volume were carefully retrieved, without resuspending the loose pellet, and mixed with 3 ml of ethanol. Samples in this condition can be safely stored at - 20 °C, until Evans Blue quantitative analysis.

A HPLC pump was interconnected to an injection port and directly connected to a fluorescence detector (Beckman®). Excitation wave length was set at 620 nm (band width 10 nm), and emission wave length 680 nm (band with 10 nm). The pump was set at 2 ml/min, delivering 50 % trichloroacetic acid : methanol (25 : 75,v/v). Aliquots of 10 µl volume were injected every 3 min into the injection port. Data was captured with a System Gold software version 6.0 (Beckman®). Evans Blue standards were solubilized in 50 % trichloroacetic acid : ethanol (25 : 75,v/v). The standard curve was run in a range of 1 to 50 ng.

Data are expressed as µg brain Evans Blue / mg proteins in the pellet divided by µg plasma Evans Blue / ml plasma.

Tissue dissection, RNA extraction and Northern blot analysis.

Brain tissue was rapidly dissected on an ice cold dissection board, and brain hippocampus was rapidly removed. Total RNA from brain regions was isolated following the guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987). Tissue was homogenized with a Polytron type homogenizer in 4 ml buffer containing 4 M Guanidine Thiocyanate, 25 mM sodium citrate, 0.5 % n-lauroyl sarcosine, and 0.1 M mercaptoethanol, pH 7.0. After precipitation, the just-purified RNA extract was resuspended in DEPC (diethyl-pirocarbonate) treated water, and an aliquot was quantified by spectrophotometric detection in a range 220 to 300 nm. Gel electrophoresis of RNA (5 µg per lane) was performed under denaturing conditions on a 1.2 % agarose gel. RNA was transferred to Hybond-N^+ nylon Membranes (Amersham, Arlington Heights Illinois), followed by hybridization at 42 °C with ^{32}P-labeled DNA probes for *Zif*-268, Tis-10, c-*fos*, *jun*-b, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ^{32}P-DNA probes were obtained by random primer extension from cDNA inserts of c-*fos* (Curran et al., 1987), zif-268 (Millbrandt, 1987), Tis-10 (Kujubu, and Herschman, 1991) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH),(ATTC # 57090), (Tso et al., 1985). Autoradiography or phosphor-image quantification were performed on a

Biorad® instrument.

Isolation of nuclei from neocortical and hippocampal tissues.

All operations were done at 4°C on wet ice, using proteolytic enzyme inhibitors phenylmethylsulfonyl fluoride (PMSF, Sigma; 1 mM), aprotinin (Sigma; 0.05 ug/ul) and leupeptin (Sigma, 0.025 ug/ul). The neocortical or hippocampal tissue mass was weighed (typically 0.2 - 1 mg wet weight) and a Kontes cordless hand held mini homogenizer (Cat.No. 749540) was used to homogenize the cells in ~500 ul of Dulbeccos Phosphate Buffered Saline (PBS) containing 1 mM PMSF and 0.05 ug/ul aprotinin, in a 1.5 ml Eppendorf vial, until suspension was completely homogeneous.

This suspension was pelleted at 4°C by centrifugation at 1400 x Gav for 10 minutes (3500 rpm in an Eppendorf 5403 centrifuge) to get rid of blood vessels, extracellular debris, etc. After carefully removing the supernatant, the pellet was gently resuspended in 500 ul of NUCLEI PREPARATION BUFFER (20 mM HEPES; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1mM DTT; 0.5 mM PMSF; 1% (v/v) aprotinin; 0.6%, v/v Nonidet P-40). 500 ul of buffer was required per single hippocampus, in each 1.5 ml Eppendorf tube. Nuclei were pelleted at 2600 x Gav (5 Krpm in an Eppendorf 5403 centrifuge) for 4 min and supernatant was removed. An aliquot of the suspension was stained with 0.1% crystal violet and observed under the microscope. Samples were kept frozen at -20°C until use.

Preparation of rat brain nuclear protein extracts (NPXTs).

To prepare NPXTs from these isolated nuclei, the pelleted nuclear fraction was resuspended in 50 - 100 ul of ice cold NUCLEI LYSIS BUFFER (20 mM HEPES; 0.4 M NaC; 11 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; 1% (v/v) aprotinin; 10% (v/v) glycerol) and vortexed intermittently for 30 minutes. In some cases, and for a higher NPXT yield, a mini-homogenizer was employed for a more thorough lysis. This lysate was microcentrifuged at 12000xG for 10 minutes (10.5 Krpm for 10 min, Eppendorf 5403 centrifuge) and the supernatant was aliquoted into new microcentrifuge tubes, supplemented with 0.025 ug/ul of leupeptin (Sigma L-2884) and stored at -81°C.

Electrophoretic Mobility Shift Assay (EMSA) and Data Analysis

NPXTs derived from control and experimental animals were subjected to electrophoretic mobility shift assay (EMSA) following the protocols of Fried and Crothers (1981) as modified by

Lukiw et al (1994). Each of the four DNA oligonucleotides, obtained from Santa Cruz Biotechnology or synthesized at the LSUMC Core Facility (Table 1), were end-labelled using gamma-^{32}P-ATP (Amersham) and were reacted with 0-5 ug of respective NPXTs in a protein-DNA assembly buffer (final volume 10 ul) consisting of 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl,), 0.05ug/ul poly dIdC and 4% (v/v) glycerol. Reactions were carried out for 30 - 60 minutes at 0-4°C. 2ul of a buffered bromophenol blue / glycerol tracking buffer was added and samples were analyzed on a 5% acrylamide Tris-Borate-EDTA (TBE) gel system (Biorad Mini Protean II system) using 0.25X TBE as running buffer, for 45 minutes at 150 V, 25 mA at 23°C. At this point the bromophenol blue had just migrated to the bottom of the gel. Gels were dried onto Whatman No. 1 filter paper (Biorad gel dryer model 583), exposed to radiographic screens, and the resulting signals were analyzed on a GS-250 Molecular Imager (BioRad). Relative intensities of gel-shifted species were quantitated using the phosphorimager analysis package available on the GS-250 Molecular Imager.

Protocol for PGHS-2 Western-blotting rat brain tissues:

Tissue samples were kept in ice until homogenization. Hippocampus tissue was rapidly homogenized in a 2 ml glass/glass homogenizer with 0.5 ml of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 5mM EGTA, 1 % Triton-X-100, pH 7.4). Protease inhibitors were added just before use: 0.1M PMSF, 0.1TIU/ml Aprotinin, and 0.1M Leupeptin; the buffer was at room temperature. Homogenates were sonicated for 30 seconds, protein concentration was measured, and samples were diluted to a concentration of 20 µg proteins in 10 µl lysis buffer. To each sample was added equal volume of 2 X concentrated electrophoresis sample buffer (250 mM Tris-HCl, pH 6.5, 2 % SDS, 10 % glycerol, 0.006 % Bromophenol Blue, and 100 µl/ml β-Mercaptoethanol). Samples were kept on ice but were boiled for 3 - 5 minutes just before gel loading,

The electrophoresis apparatus assembly and gel casting were started before tissue homogenization. Samples were loaded (20 µl per well) onto a 0.75 - 1.00 mm thick SDS-polyacrylamide gel and the gels were run at 180 to 200 volts, for about 45 to 60 minutes. For gel transfer, the gel was soaked in ice cold transfer buffer (4°C) for 30 min (to allow the gel to shrink to the final size and remove residue of running buffer). The gels and nylon membrane were installed on transfer apparatus, and the transfer was done at 100 V for about 1 hour. The

membranes were removed from the cassettes, the position of main bands from molecular markers was marked, and membranes were soaked in blocking buffer on an oscillatory platform overnight at 4 °C, or for 30 minutes at 37 °C. They were then incubated at 37 °C with the primary antibody for 30 minutes. The primary antibodies were diluted in 10 ml of blocking buffer as follows: PGHS-1: **1/250** (Monoclonal Anti-PGHS-1) Cat.# PG21 Oxford Biochemical Research; PGHS-2: **1/2500** (Monoclonal Anti-PGHS-2) Cat.# C22420 Transduction Laboratories. The membranes were washed in fresh blocking buffer and placed in weighing buckets with 25 ml blocking buffer at room temperature, on a rocking platform. Each 5 min., the buffer was replaced, and was kept rolling for 30 minutes. Membranes were then incubated with a secondary antibody at 37 °C for 30 minutes in the rotatory oven. Dilution of the secondary antibody in 10 ml of blocking buffer was as follows: Anti-Mouse IgG: **1/2000** (Anti-Mouse IgG horseradish peroxidase conjugate) Catalog # M15345L3. Transduction Laboratories; for alkaline phosphatase we used **1/10,000** dilution of the secondary antibody. The membranes were washed in the blocking buffer, and placed in weighing buckets with 25 ml blocking buffer at room temperature, on a rocking platform. Each 5 minutes the buffer was replaced, and was kept rolling until completed (30 minutes.)

Enhanced chemiluminescence detection (ECL, Amersham)

This procedure was followed for horseradish peroxidase conjugated secondary antibodies. The buffer was wiped off with 3M paper and the membranes placed on Saran-Wrap, taking care to place the marked side up (where proteins are bound). Equal volumes of detection solutions 1 and 2 were mixed and overlied on top of membranes which then were incubated for 1 minute. Excess of solution was then wiped-off . The membranes were covered with a layer of Saran-Wrap and exposed to autoradiography film (Hyperfilm-ECL) for 1 to 5 minutes.

Enhanced chemiluminescence detection for Alkaline-phosphatase (Western-light™, Tropix).

The membranes were washed twice with Assay Buffer for 2 minutes each, incubated with 5 ml of Assay Buffer containing 0.25 mM CSPD, 1:20 Nitro-Block:AssayBuffer and exposed to autoradiography film (Hyperfilm-ECL) or phosphoimager plate. Chemiluminescence reaches its maximum intensity for this reaction kit 1 hour after activation.

Results.

A) Nuclear protein transcription factors in the rat brain after cryiogenic injury (EMSA analysis)

The characteristics of the nuclear protein transcription factors AP2, CREB, GAS/ISRE and NFκB are outlined in **Table 1a**, and the DNA sequences of their binding sites are outlined in **Table 1b.**

Table 1a: Transcription Factors Quantitated in the Rat Cortex and Hippocampus after Cryogenic Brain Injury

Transcription Factor	Tissue Specificity	Inducers	Repressors	Features
Synthesized *de novo*:				
AP2 (activator protein 2) **CREB** or (cyclic AMP responsive element)	most abundant in the neural crest lineage	TPA, cAMP, retinoic acid	SV40 Tag DNA methylation	Also binds to SP1, NF1 and SV40 TAg binding sites can bind as homo heterodimers
Activated from *latent* factors:				
NFκB (nuclear	ubiquitous factor kappa-B)	cAMP, IL1, IL6 lipopolysaccharide	-	Binds as a dimer hetero or tetra-dimer
GAS/ISRE (STAT1α, 1β and 2) (signal transducer & activators of transcription)				released after stimulation

Table 1b: Transcription Factor DNA Binding Consensus Sequences Utilized in these Studies

AP2 CONSENSUS OLIGONUCLEOTIDE
5'-GATCGAACTGACCGCCCGCGGCCCGT-3'
Source: LSUMC Core Labs

CREB CONSENSUS OLIGONUCLEOTIDE
5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3'
Source: Promega, Santa Cruz Biotechnologies

GAS/ISRE CONSENSUS OLIGONUCLEOTIDE
5'- AAG TAC TTT CAG TTT CAT ATT ACT CTA -3'
3'- TTC ATG AAA GTC AAA GTA TAA TGA GAT -5'
ISRE CONSENSUS OLIGONUCLEOTIDE
5'- AAG TAC TTT CAG TTT CAT ATT ACT CTA -3'
3'- TTC ATG AAA GTC AAA GTA TAA TGA GAT -5'
Source: Santa Cruz Biotechnologies

NFKB CONSENSUS OLIGONUCLEOTIDE
5'-AGTTGAGGGGACTTTCCCAGGC-3'
Source: Santa Cruz Biotechnologie

The results from a typically run EMSA 5% acrylamide, 0.25 x TBE gel scan using the DNA consensus sequences for NFκB and GAS/ISRE are shown in **Figure 1A and IB,** respectively.

FIGURE 1A

A TYPICAL EMSA EXPERIMENT USING END LABELLED NFKB DNA ELEMENT AND NPXTs DERIVED FROM CRYOGENICALLY INURED RAT BRAIN

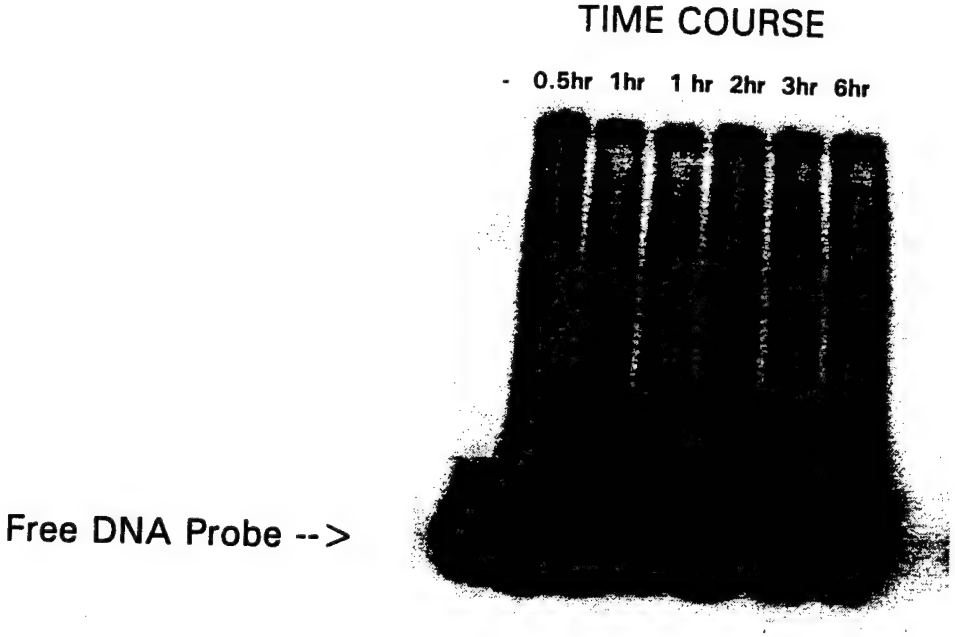
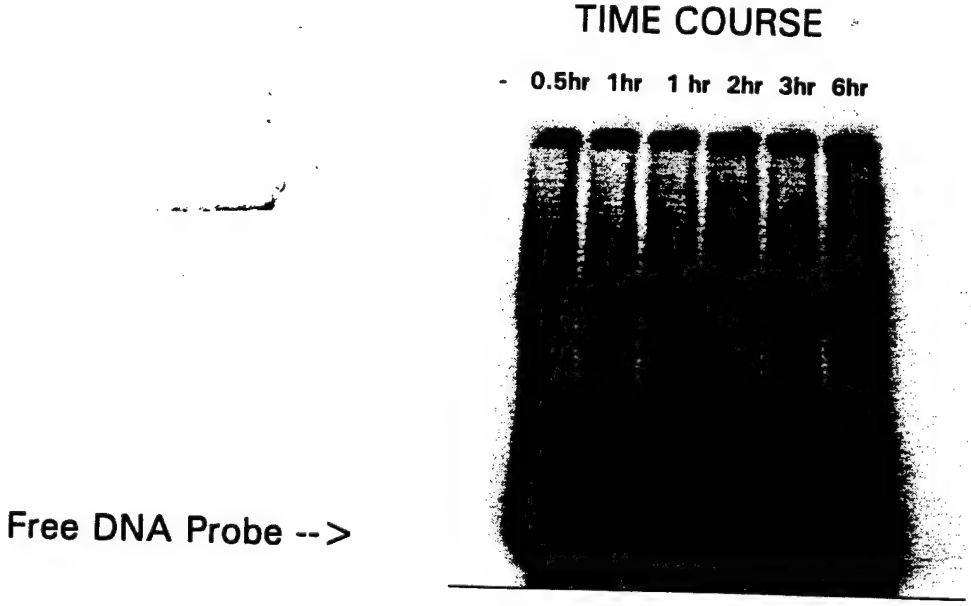


FIGURE 1B

A TYPICAL EMSA EXPERIMENT USING END LABELLED GAS/ISRE DNA ELEMENT AND NPXTs DERIVED FROM CRYOGENICALLY INURED RAT BRAIN



The levels of NFκB, GAS/ISRE, AP2, and CREB protein-DNA binding in **the rat brain hippocampus** over a time course of 0-6 hours after cryogenic injury are shown in **Figure 2**.

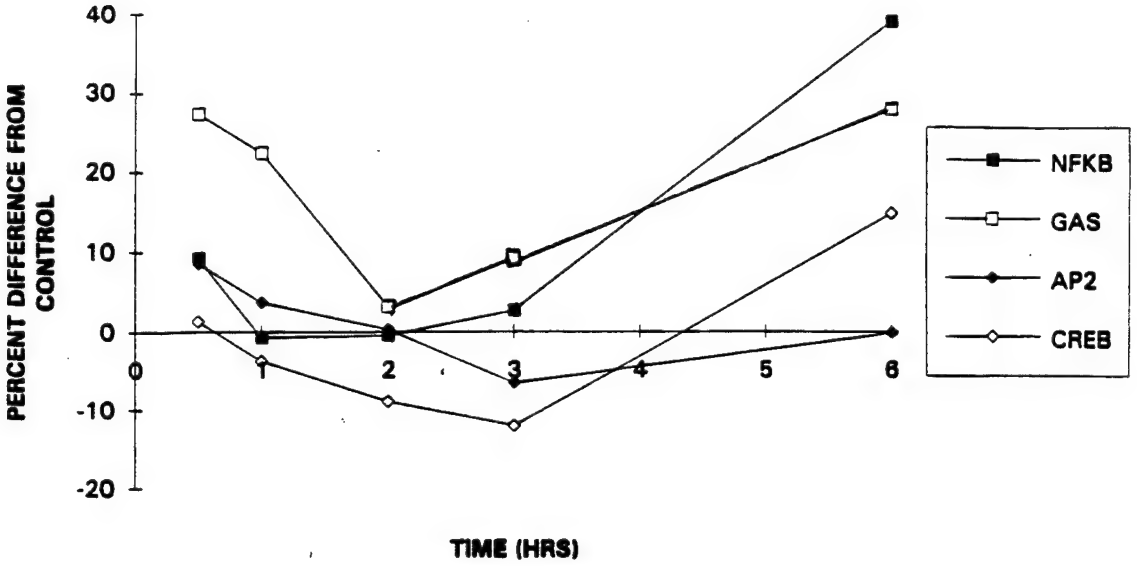


Figure 2. Transcription factor time course in the **rat hippocampus** after cryogenic injury.

The levels of NFκB, GAS/ISRE, CREB, and AP2 protein-DNA binding in **the rat brain cortex** over a time course of 0-6 hours after cryogenic injury are shown in **Figure 3.**

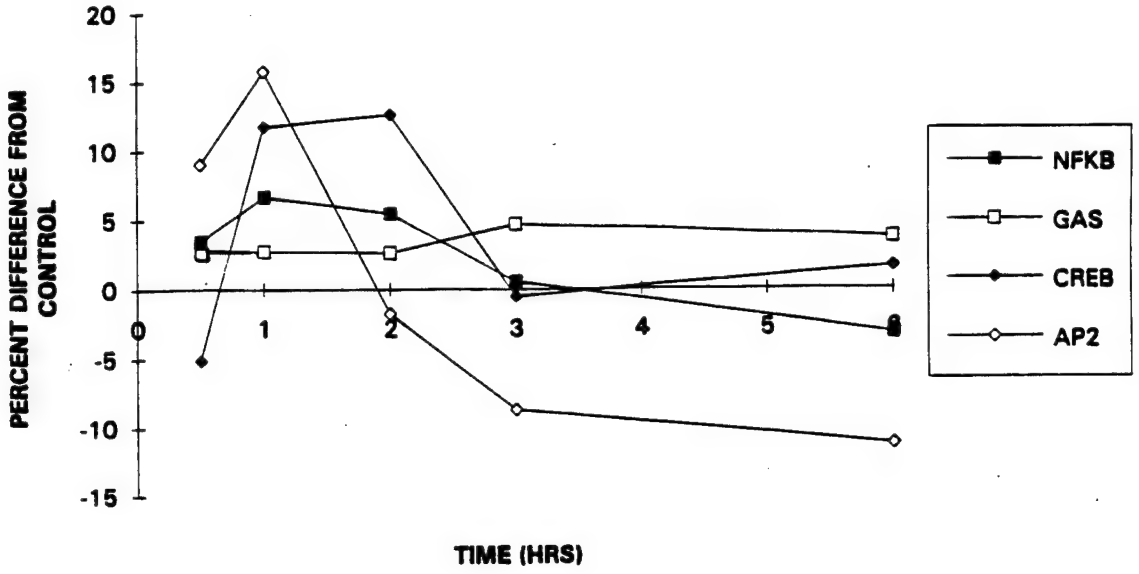


Figure 3. Transcription factor time course in the **rat cerebral cortex** after cryogenic injury.

The levels of protein binding to the GAS/ISRE response element in the ipsilateral (injured side) and contralateral **rat hippocampus** are shown in **Figure 4**.

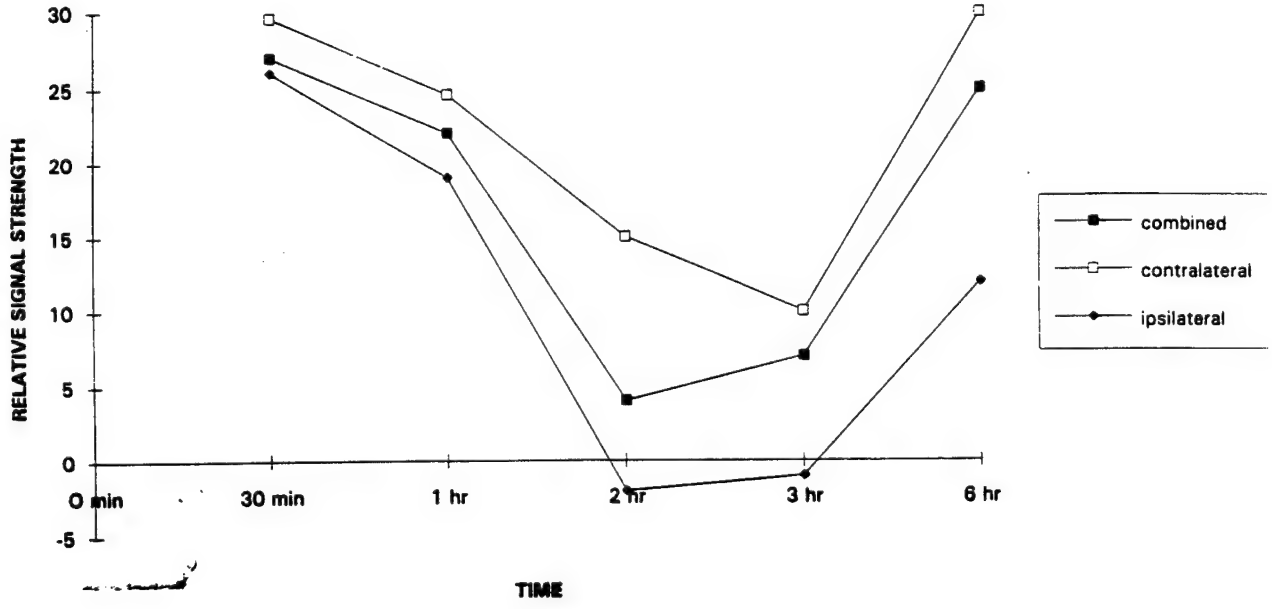


Figure 4. GAS/ISRE response element and NPXTs from **rat hippocampus** ipsilateral and contralateral to cryogenic injury.

The levels of protein binding to the GAS/ISRE response element in the ipsilateral (injured side) and contralateral **rat cortex** are shown in **Figure 5**.

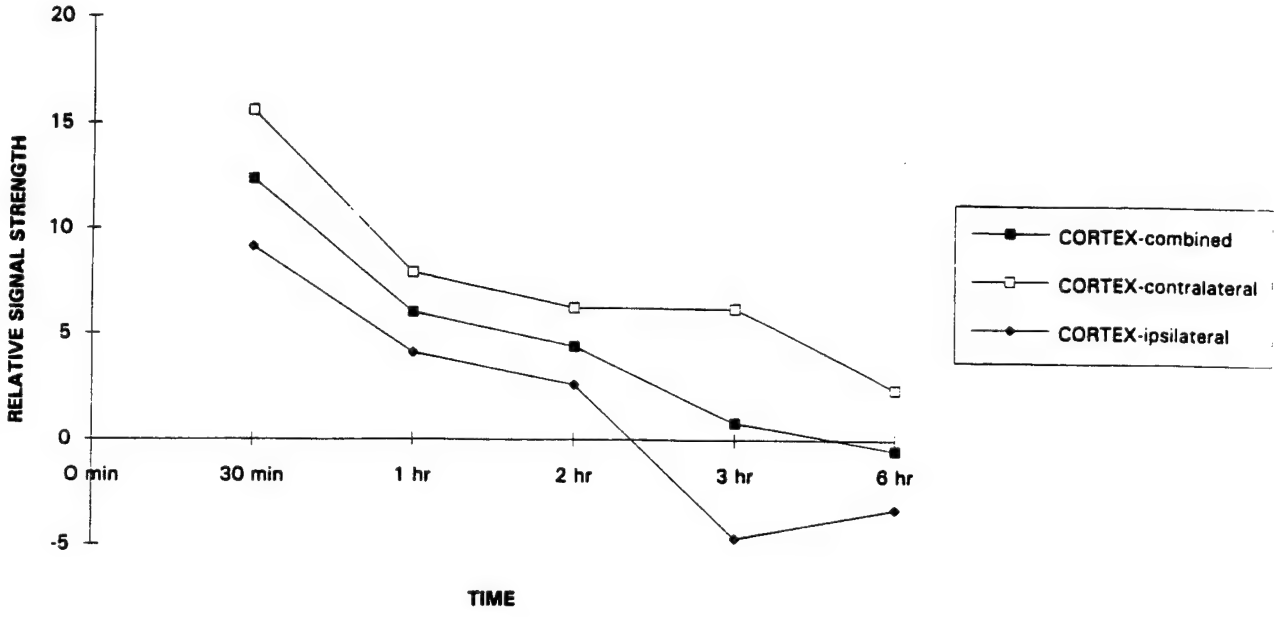


Figure 5. GAS/ISRE response element and NPXTs from **rat cortex** ipsilateral and contralateral to cryogenic injury.

The identification of a novel brain injury related protein which interacts with the COX-2 promotor region (-460 to -258 bp) in the rat is shown in **Figure 6.**

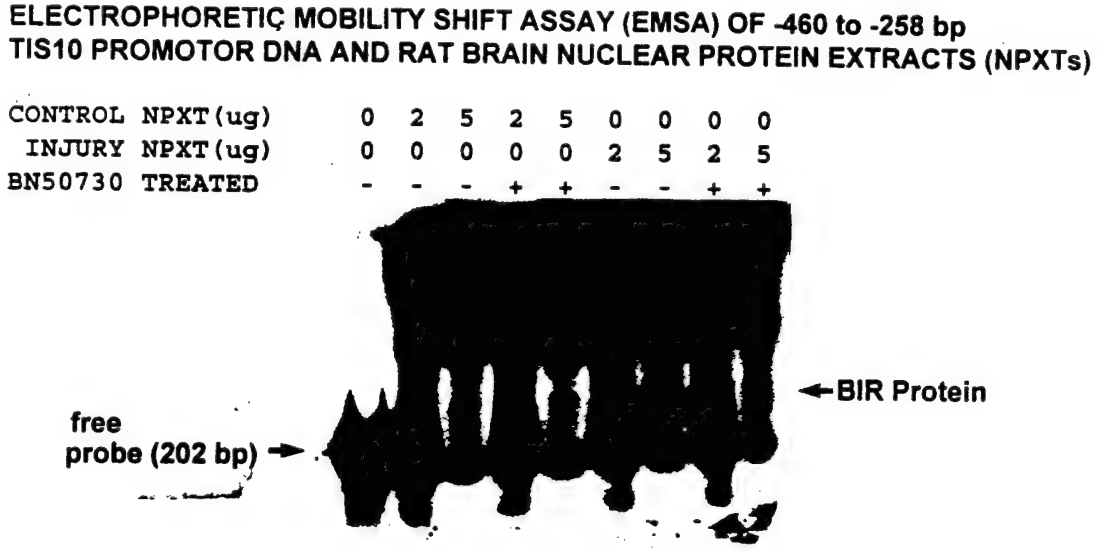


Figure 6. Shows the results of EMSA analysis using 202 bp of the TIS 10 (COX2, PGHS2) promotor DNA (-460 to -258 bp) and nuclear protein extracts (NPXTs) derived from control and cryogenically injured rat hippocampal nuclei. A brain injury related (BIR) gel shift (arrow) was detected using NPXT's derived from injured rat hippocampal nuclei in the absence of PAF antagonist BN50730.

The oligonucleotide competition assay, to show that this brain injury related protein (BIR) is related to AP2 or NFκB, is shown in **Figure 7.**

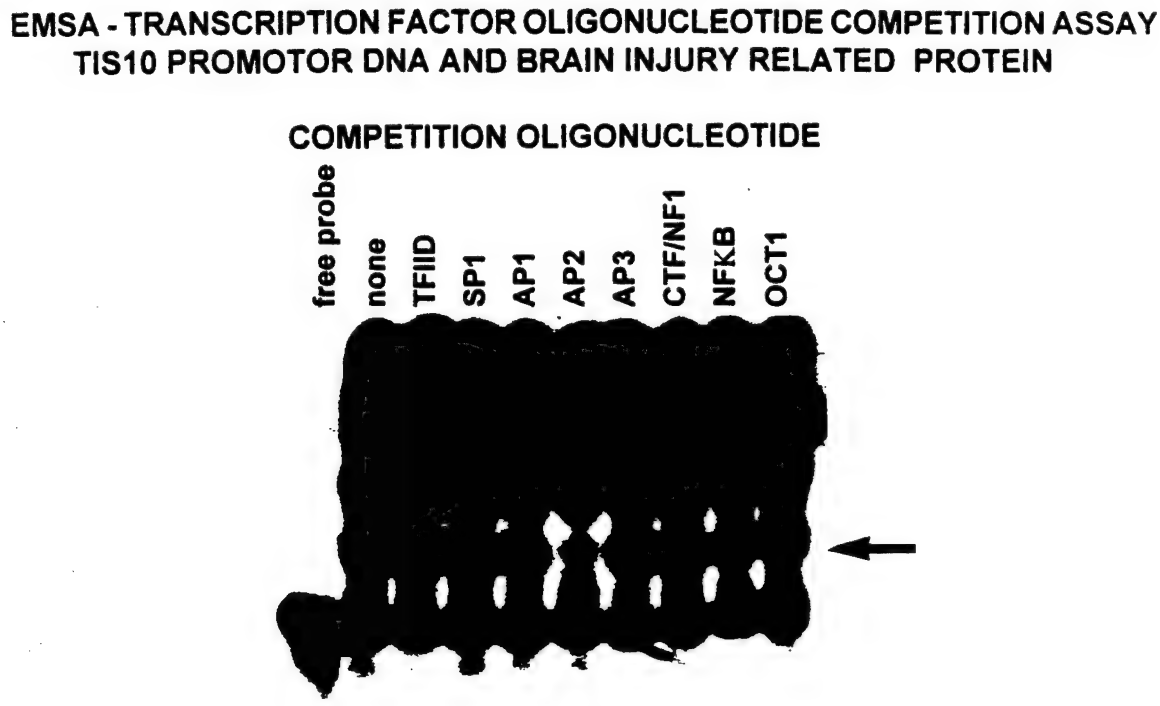


Figure 7. Shows the results of a transcription factor oligonucleotide competition assay wherein oligomers bearing the DNA-binding consensus sequence for 8 transcription factors were preincubated with the EMSA assay as shown in Figure 6. A strong reduction of signal of the BIR gel-shift upon preincubation with the competition oligonucleotides AP2 and NFκB (arrow) implicate these two transcription factors in the regulation of TIS-10 (COX-2, PGHS-2) gene induction during brain injury.

B) PGSH-2 protein levels in the brain after cryogenic injury (Western Blot analysis)

Changes in PGSH-2 protein levels in the rat hippocampus were analyzed by Western Blot at different times after cryogenic injury **(Figure 8)**. The enzymatic protein reached maximal values by 4-5 hours after injury, decreasing thereafter.

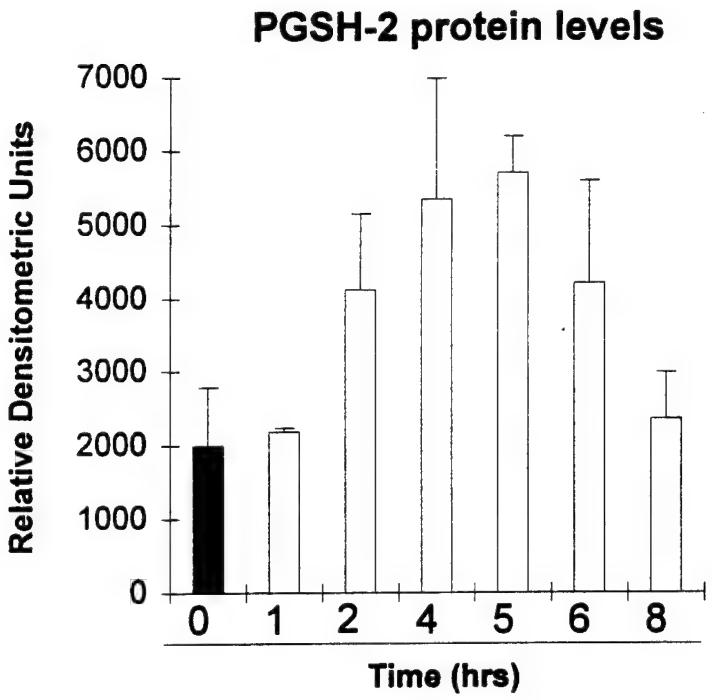


Figure 8 Rat hippocampus PGS-2 protein levels after cryogenic injury. Samples were analyzed by Western Blot. Bars represent mean values ± S.D. from 4-5 samples, except for 1 and 8 hour time points, which represent mean values ± dispersion from the mean (n=2). Black bar: control prior to cryogenic injury. Values at 2, 4, 5, and 6 hours after cryogenic injury are statistically significantly different from control ($p<0.05$, Student's *t* test).

C) A new PAF antagonist that protects the brain from cryogenic injury-induced edema (Evans blue extravasation and Northern Blot analysis)

1- *Validation of LAU as a PAF receptor antagonist*: The displacement of [^{3}H]PAF binding by a new PAF antagonist LAU-503 on microsomal membranes is shown in **Figure 9.** Analysis of the data gave an IC_{50} = 1.904 ± 0.470 pM and an K_i (equilibrium dissociation constant) = 1.889 ±0.467 pM. The results demonstrate that LAU-503 is a potent non competitive or uncompetitive ($IC_{50} = K_i$) inhibitor of PAF binding to intracellular binding sites.

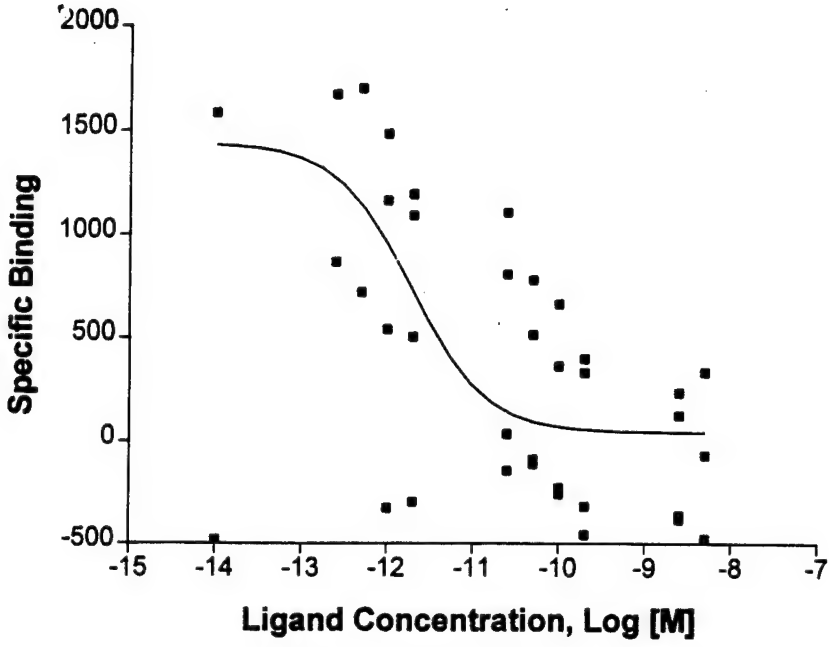


Figure 9. [^{3}H]PAF displacement in microsomal membranes by LAU-503. Increasing concentrations of LAU-503 were used to displace 3nM [^{3}H]PAF after 30 min at 25° (Marcheselli et al., 1990). Data were obtained by interacting results from four experiments using an EBDA-LIGAND program. The overall analysis of the curve gave a p value <0.0001.

2- *Effect of LAU-503 on brain edema*: The effect of LAU-503, dexamethasone, and dexamethasone+LAU-503 on cryogenic injury-induced vasogenic edema in the rat brain was studied by measuring the levels of Evans Blue extravasation in the right (damaged) and left (contralateral) cerebral cortex (**Figure 10**). By 1 hour dexamethasone, but not LAU-503, inhibited by 100% Evans Blue extravasation. By 12 hours, LAU-503 inhibited by about 50% edema formation while dexamethasone gave values similar to sham animals (100% protection).

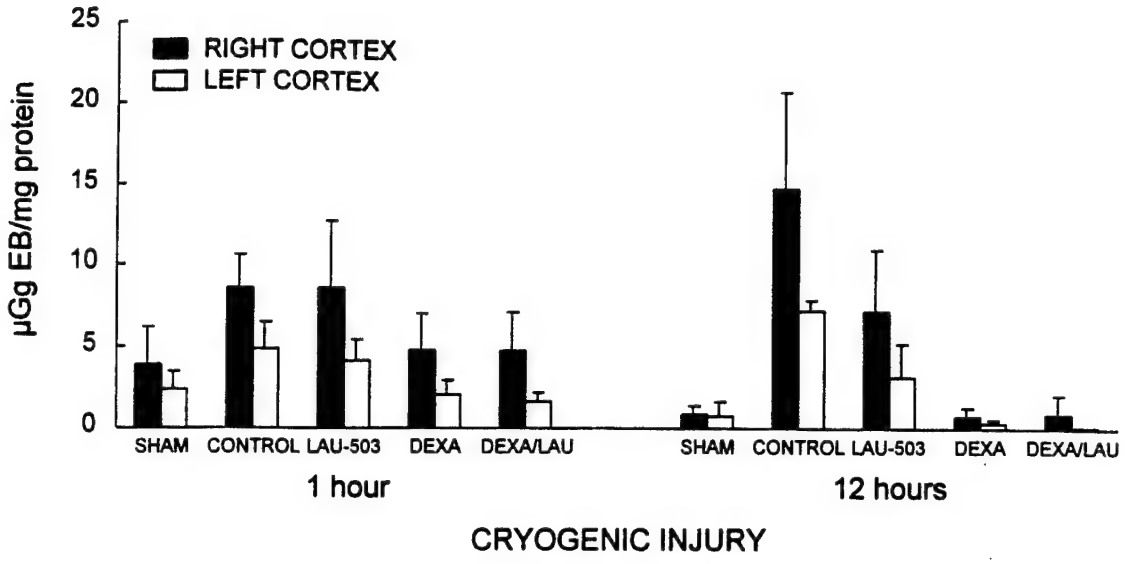


Figure 10. Evans Blue extravassation after cryogenic brain injury. Rats were *iv* injected with 2 ml of Evans Blue (2% in saline solution) one hour before cryogenic injury. Animals were injected *icv* with the vehicle (DMSO, sham) or LAU-503 in both ventricles (30 µg/animal). Dexamethasone treated rats received 3 *ip* injections (6.7 µg/µl, 50 µl) every other 8 hours. One and 12 hours after the injury animals were killed, the right (damaged) and left (contralateral) cortex dissected and homogenized in 50% trichloroacetic acid. An aliquot of 10 µl was injected in the HPLC system and run with a solution containing trichloroacetic acid 50%:methanol (25:75, v/v) at 2 ml per min. The system was connected to a fluorescence detector. Excitation and emission wave lengths were 620 nm and 680 nm, respectively.

3- *Effect of LAU-503 on brain levels of mRNA TIS-8 (Zif-268)/TIS-10* (COX-2) *after cryogenic injury:* The effect of LAU-503 on cryogenic injury-induced TIS-10 mRNA expression is shown in **Figure 11.** The effect of this new PAF antagonist is compared with that of the antagonist BN50730 (shown in the previous report) and dexamethasone. TIS-10 mRNA levels were increased in non treated animals 8.3- and 3.5-folds at 2 and 6 hours, respectively, after injury. Two hours after the injury, BN50730 was the more potent inhibitor of TIS-10 expression (-65.7% inhibition, $p<0.00004$) followed by LAU-503 (-48.6%, $p<0.00005$) and dexamethasone (-40.2%, $p<0.00007$). By 6 hours, LAU-503 showed greater inhibitory effect (-55.6%, $p<0.000006$) than BN50730 (-49%, $p<0.000005$), and dexamethasone did not affect TIS-10

expression.

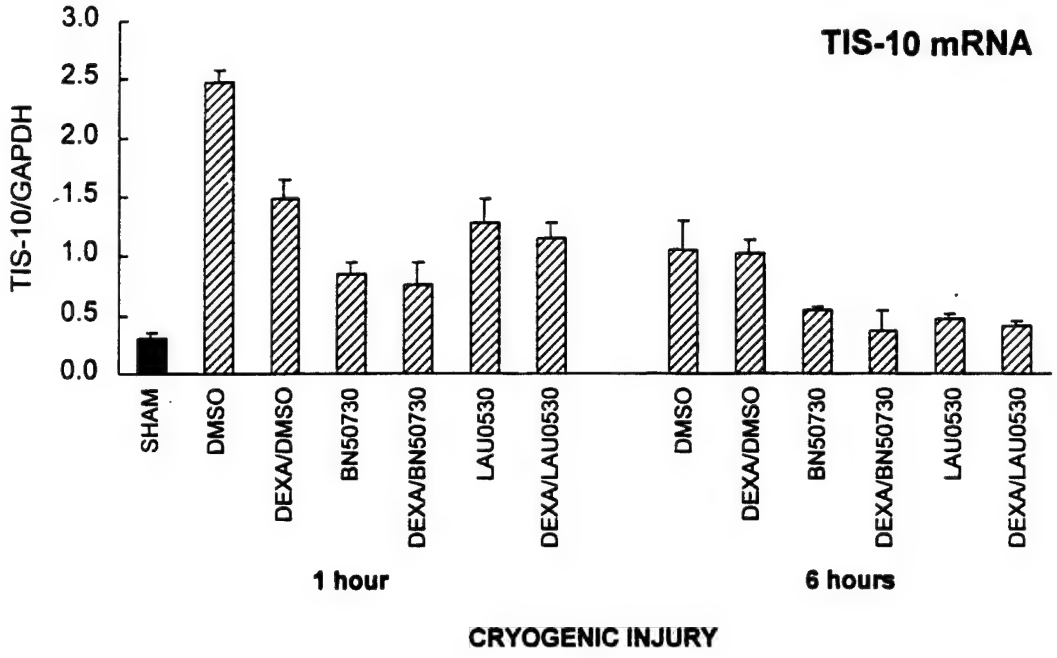


Figure 11. Brain expression of **TIS-10 mRNA** after cryogenic injury: Effect of PAF antagonists LAU-503 and BN50730 and dexamethasone. Two and 6 hours after cryogenic edema, right brain cortex (damaged) was dissected, mRNA extracted and Northern Blot analysis performed. Blots were hybridized for TIS-10 mRNA. The gene was screened in conjunction with GAPDH as a housekeeping gene. Data obtained by phosphor-image quantification was normalized with GAPDH mRNA. Other details as in Fig. 10 legend.

The effect of LAU-503 on cryogenic injury-induced TIS-8 mRNA expression is shown in **Figure 12.** TIS-8 mRNA levels were increased 2.8-fold 2 hours after injury and were already at basal (sham) levels after 6 hours. By 2 hours after injury, levels of TIS-8 mRNA decreased significantly by BN50730 (-60.8%, $p<0.0007$), followed by LAU-503 (-46.6%, $p<0.04$) and dexamethasone
(-36.5%, $p<0.05$).

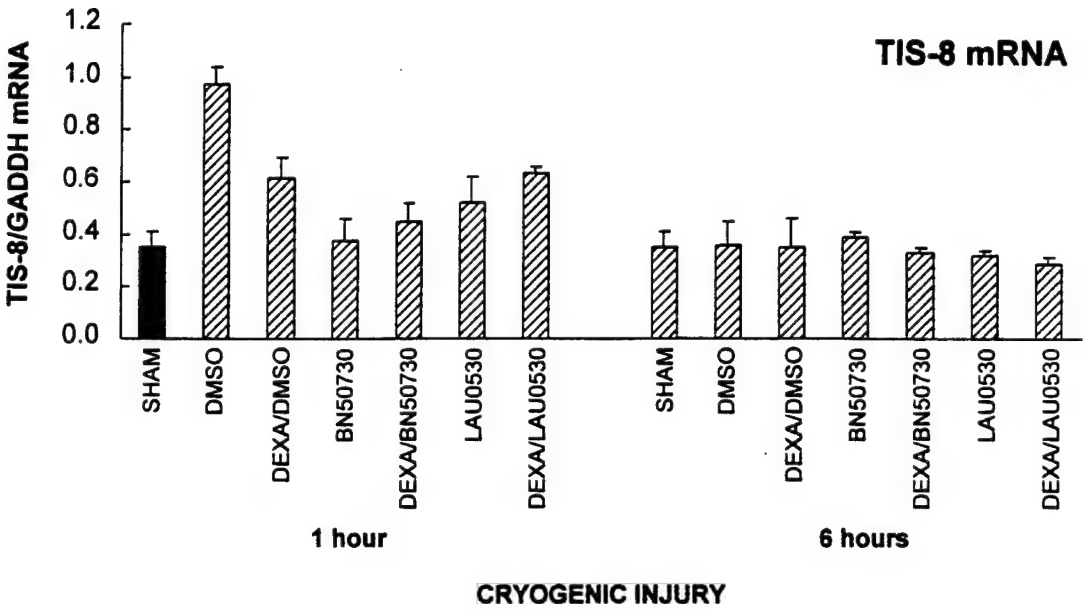


Figure 12. Brain expression of **TIS-8 mRNA** after cryogenic injury: Effect of PAF antagonists LAU-503 and BN50730 and dexamethasone. Northern blots were hybridized for TIS-8 mRNA. Other details as in Fig. 11 legend.

SUMMARY AND CONCLUSIONS

A) A novel DNA binding protein which interacts with the TIS10 promotor region (-460 to -258 bp) was identified in rat brain nuclear protein extracts. This protein was present only in the control state, and disappeared during the course of cryogenic injury. We speculate that one mechanism responsible for the activation of the COX-2 gene, and its role in the inflammatory response, may be an alleviation of repression during the cryogenic injury event. This may represent one important endpoint in the cellular signal transduction pathways by which events occuring at the brain cell periphery are relayed to the nucleus to yield primary genetic responses to cryogenic injury.

1. There are specific interactions of NPXT's, which are derived from both cryogenically injured cortex and hippocampus, and contain DNA consensus sequences for AP2, CREB, GAS/ISRE and NFκB.

2. There is a tendency for each of these transcription factors to decrease in abundance over a time course of 0-6 hours after cryogenic injury. The decrease in the rat hippocampus precedes that measured in the rat cortex.

3. Ipsilateral/contralateral sides of brain injury - experiment in progress.

4. A number of immediate early genes are induced following cryogenic injury. In the promotor of a gene coding for the inducible form of cyclooxygenase II (COX-2), a novel binding protein was found bound to the -460 to -258 bp region in the resting (control) state. However, during gene activation following cryogenic injury, the level of this protein is dramatically reduced. This novel protein may act as a gene repressor, which when released after cryogenic and other forms of brain injury, may activate the COX-2 gene and potentiate the role of this gene in brain inflammation.

B) A specific increase in the PGSH-2 protein levels was also obtained at 4 to 5 hours after injury two to three hours after an increase of the COX-2 (PGSH-2) mRNA levels induced by cryogenic injury (Previous report, **year 1).**

C) LAU-503 is an intracellular PAF antagonist that inhibits injury-stimulated TIS-10/TIS-8 mRNA, further supporting the role of PAF as an injury-mediator of early gene expression.

1- Kinetic studies using the new PAF antagonist LAU-503 revealed that this drug is a potent non competitive inhibitor of PAF binding to microsomal membranes with an IC_{50} = 1.2 pM, 1000 times smaller than that of BN50730 (IC_{50} = 1.2 nM).

2- LAU-503 inhibited edema formation by 50% at 6 hours after injury.

3- LAU-503 inhibits the injury-induced expression of TIS-10/TIS-8 mRNA genes. By 6 hours the inhibitory effect of LAU-503 was significantly higher than that of BN50730.

Research plan for the upcoming year

1- As suggested by the reviwer, the traumatic brain injury model developed in our laboratory to injure the brain at low, medium, and severe pressure, will be validated by histological analysis of the tissue. This work is currently in progress.

2- Analysis of free fatty acids and diacylglycerides will be performed at different times after traumatic brain injury.

3- Analysis of early gene expression and the effect of PAF antagonists will be performed following experimental protocols similar to the ones used after cryogenic injury.

4- Synaptosomes isolated from injured animals will be used to follow *in vitro* the stimulatory effect of PAF on [^{3}H]glutamate release.

Abstracts and publications from this work.

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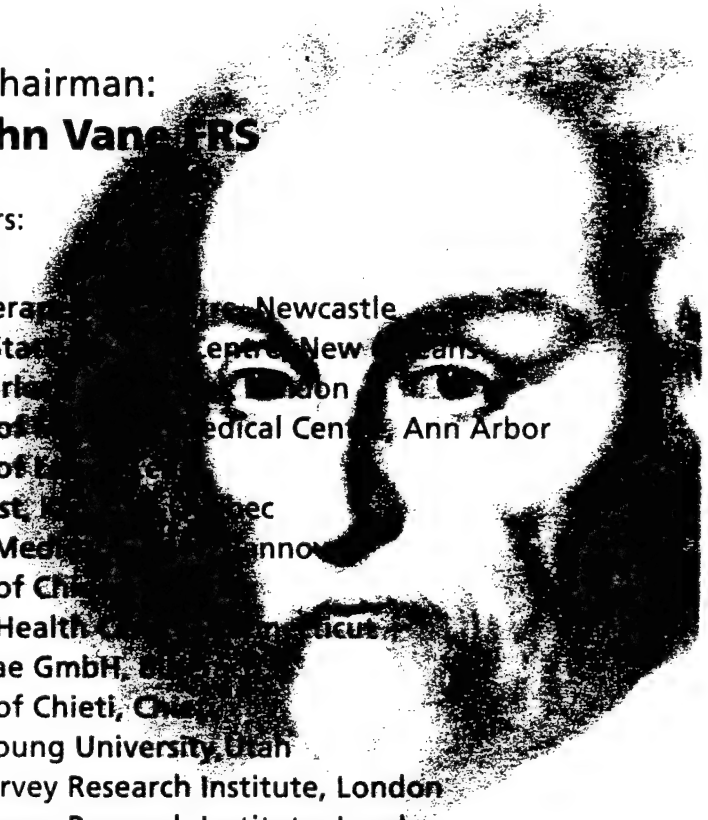

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An inhibitor of injury-induced COX-2 transcriptional activation elicits neuroprotection in a brain damage model

by NICOLAS G BAZAN and VL MARCHESELLI

Louisiana State University Med. Center, Neuroscience Center of Excellence, New Orleans, LA, USA

Brain injury triggers the rapid activation of phospholipase A_2 (PLA_2) (Bazan, 1970) with the accumulation of free arachidonic and docosahexaenoic acids and of lyso-platelet-activating factor (lyso-PAF), the precursor of PAF (Bazan et al, 1995). In ischemia-reperfusion injury or in seizures a burst of eicosanoids synthesis follows PLA_2 activation (Bazan, 1995), Alternative pathways of PAF, metabolism (CoA-independent transacylase and the *de novo* route of synthesis) remain to be explored and compared with PLA_2 as sources of PAF generated in brain injury.

PAF is a transcriptional activator of COX-2 (Bazan et al,1994) since it activates the expression of luciferase reporter constructs driven by regulatory regions of the COX-2 gene. Induction is rapid and is inhibited by BN 50730, suggesting that pre-existing latent transcription factor/s are involved. Deletion studies restrict the major PAF cis-acting response element to a 70 nucleotide sequence (in the case of the mouse COX-2 promoter) as an intracellular inducer of COX-2 expression.

In a vasogenic model of cerebral edema, there is a sustained upregulation of COX-2 transcription as measured by nuclear run-on assays, mRNA abundance and Western blots of enzyme protein. COX-1, the constitutive isoform of the enzyme remains unchanged. Thus COX-2 enhanced expression is consistent with the prediction that this inducible enzyme is part of the brain inflammatory-injury response. PAF accumulates in the brain during injury. Two inhibitors of COX-2 were studied, dexamethasone (given prior to injury) and BN 50730 (given once by intracerebroventricular injection 1 hr prior to injury). The PAF antagonist BN 50730 inhibited COX-2 expression in brain 65% and 58% at 2 and 6 hrs after injury, respectively. Dexamethasone inhibited COX-2 to a lesser extent than BN 50730, with a 40% and 35% inhibition at 2 and 6 hrs, respectively. Neuroprotection measured by Evans blue quantitative assessment by fluorescence measurements both in the blood and in the brain indicate that BN 50730 prevented damage by 40% and 67.2% at 1 and 12 hrs after injury, respectively.

The injury-induced increase in COX-2 expression may elicit cellular damage through a number of mechanisms including the generation of O^-_2 in the conversion of PGH_2 to prostaglandins. This PLA_2 ischemic injury cascade may participate in oxidative stress during the pathophysiology of head injury, epileptic brain damage (status epilepticus), in some neurodegenerative diseases (e.g. HIV-related neuronal injury), and in ischemia-reperfusion, both of the brain (e.g. stroke) and of non-neural organs (i.e. myocardium and kidney).

It is concluded that a) injury-induced COX-2 transcriptional upregulation is involved in brain damage, b) PAF is a mediator of brain injury-induced COX-2 expression, c) BN 50730, an intracellular inhibitor of PAF genomic effects, blocks brain injury-induced COX-2 expression, and d) the latter, at the same time, elicits neuroprotection in a model of vasogenic cerebral edema.

Supported by DAMD 17-93-V-3013.

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EFFECT OF SECRETORY PHOSPHOLIPASES A_2 AND GLUTAMATE ON VIABILITY OF RAT CORTICAL NEURONS AND CALCIUM DYNAMICS. M. Kolko, M. A. DeCoster*, G. Lambeau, M. Lazdunski and Nicolas G. Bazan. Neuroscience Center, LSU Medical Center, New Orleans, LA 70112-2234.

Nonpancreatic secretory phospholipases A_2 (sPLA$_2$s) may be involved in modulation of neuronal function. A receptor binding sPLA$_2$s has been cloned from muscle (Lambeau, et. al. JBC, 1994). Two sPLA$_2$s from snake venom (OS_1 and OS_2) bind to this receptor. OS_2 and sPLA$_2$ from bee venom (BV) bind avidly to brain membranes, while OS_1 does not. We evaluated the neurotoxicity of OS_1, OS_2 and BV combined with glutamate (GLU). Lactate dehydrogenase (LDH) release was used as the toxicity assay. BV and OS_2 dose dependently (0.01-10 µg/mL) caused neurotoxicity and OS_1 did not. GLU (80 µM) was as toxic as approximately 0.5 µg/mL BV. Submaximally toxic concentrations of BV combined with GLU demonstrated higher toxicity levels than the two compounds added separately. BV (0.05 µg/mL) and GLU together showed three times more toxicity than the sum of the separate toxicity levels, indicating that GLU and BV are having a synergistic effect. The toxicity experiments were combined with studies investigating the effect of the sPLA$_2$s on intracellular free calcium concentration ($[Ca^{2+}]_i$). The fluorescent calcium indicator fluo-3 was used with a confocal microscope to measure real-time calcium dynamics in these neurons. We observed basal oscillations in $[Ca^{2+}]_i$ in the cultures. BV and OS_2 dose-dependently (0.5-10 µg/mL) altered ($[Ca^{2+}]_i$) dynamics, while OS_1 had no effect. Both BV and OS_2 (0.5 - 10 µg/mL) obliterated calcium oscillations, and also decreased $[Ca^{2+}]_i$ to below baseline levels. We did not see any calcium modulations in the cultures exposed to sPLA$_2$ concentrations below 0.5 µg/mL, even though the sPLA$_2$s were toxic to about 0.025 µg/mL. These results indicate that calcium-independent toxicity may be occurring at low sPLA$_2$ concentrations. Our results support the observed binding properties of sPLA$_2$s in muscle and brain tissue, and provide evidence for modulatory roles of sPLA$_2$s in neuronal signal transduction. (Supported by DAMD-17-93-V-3013).

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3. CELL CULTURE
4. GLUTAMATE

PLATELET-ACTIVATING FACTOR INDUCED INTRACELLULAR CALCIUM OSCILLATIONS IN RAT HIPPOCAMPAL NEURONS.

M. A. DeCoster, H. E. P. Bazan*, and N. G. Bazan. LSU Medical Center, Neuroscience Center, New Orleans, LA 70112-2234

As has been previously shown, we have found using confocal microscopy and fluorescent calcium indicators, that intracellular calcium concentration ($[Ca^{2+}]_i$) oscillates spontaneously in rat hippocampal neurons *in vitro*. While addition of glutamate (GLU) to these hippocampal cultures causes distinct $[Ca^{2+}]_i$ changes ranging from transient, single spikes (100-500 nM GLU) to sustained increases (20-80 μM GLU), GLU does not appear to induce $[Ca^{2+}]_i$ oscillations. We have investigated the ability of the potent lipid mediator platelet activating factor (PAF) to affect $[Ca^{2+}]_i$ dynamics in hippocampal neurons. When 4 μM methylcarbamyl PAF (mcPAF) was added to the hippocampal neurons, the average $[Ca^{2+}]_i$ was increased slightly in cells. Furthermore, the variance of fluorescence values after mcPAF additions was 8-fold higher than before additions, indicating an increase in oscillatory $[Ca^{2+}]_i$ dynamics induced by PAF. Neurons not spontaneously oscillating were observed to be induced to oscillate by PAF addition, and neurons spontaneously oscillating increased in oscillatory behavior upon PAF addition. In agreement with Bito *et al.* (Neuron, 9:285, 1992) we found that not all neurons responded to acute PAF application. In contrast, long-term effects of PAF treatment on hippocampal cultures appeared to affect the majority of cells. Overnight treatment with PAF (200-400 nM) and mcPAF (2-4 μM) reduced the neuronal $[Ca^{2+}]_i$ changes induced by GLU the next day when compared with cells pretreated with lysoPAF (2-4 μM) or the vehicle alone. In two cases, the $[Ca^{2+}]_i$ increases in response to 500 nM GLU were completely inhibited by PAF pretreatment. Since PAF has been shown to enhance hippocampal excitatory synaptic transmission (Clark et al., Neuron 9:1211, 1992) we postulate that induction of $[Ca^{2+}]_i$ oscillations by PAF may be an early signal of GLU release, resulting in GLU receptor desensitization (Supported by DAMD-17-93-V-3013).

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JOURNAL OF NEUROTRAUMA
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Neurotrauma — phospholipase A2 - PAF, arachidonic acid, oxygenated prostaglandins - inducible prostaglandin synthetase

Mediators of Injury in Neurotrauma: Intracellular Signal Transduction and Gene Expression

NICOLAS G. BAZAN, ELENA B. RODRIGUEZ DE TURCO, and GEOFFREY ALLAN

ABSTRACT

Membrane lipid-derived second messengers are generated by phospholipase A_2 (PLA_2) during synaptic activity. Overstimulation of this enzyme during neurotrauma results in the accumulation of bioactive metabolites such as arachidonic acid, oxygenated derivatives of arachidonic acid, and platelet-activating factor (PAF). Several of these bioactive lipids participate in cell damage, cell death, or repair-regenerative neural plasticity. Neurotransmitters may activate PLA_2 directly when linked to receptors coupled to G proteins and/or indirectly as calcium influx or mobilization from intracellular stores is stimulated. The release of arachidonic acid and its subsequent metabolism to prostaglandins are early responses linked to neuronal signal transduction. Free arachidonic acid may interact with membrane proteins, i.e., receptors, ion channels, and enzymes, modifying their activity. It can also be acted upon by prostaglandin synthase isoenzymes (the constitutive prostaglandin synthase PGS-1 or the inducible PGS-2) and by lipoxygenases, with the resulting formation of different prostaglandins and leukotrienes. Glutamatergic synaptic activity and activation of postsynaptic NMDA receptors are examples of neuronal activity, linked to memory and learning processes, which activate PLA_2 with the consequent release of arachidonic acid and platelet-activating factor (PAF), another lipid mediator. Both mediators may exert presynaptic and postsynaptic effects contributing to long-lasting changes in glutamate synaptic efficacy or long-term potentiation (LTP). PAF, a potential retrograde messenger in LTP, stimulates glutamate release. The PAF antagonist BN 52021 competes for receptors in presynaptic membranes and blocks this effect. PAF may also be involved in plasticity responses because PAF leads to the expression of early response genes and subsequent gene cascades. The PAF antagonist BN 50730, selective for PAF intracellular binding, blocks PAF-mediated induction of gene expression. A consequence of neural injury induced by ischemia, trauma, or seizures is an increased release of neurotransmitters, that in turn generates an overproduction of second messengers. Glutamate, a key player in excitotoxic neuronal damage, triggers increased permeation of calcium mediated by NMDA receptors and activation of PLA_2 in postsynaptic neurons. NMDA receptor antagonists reduce the accumulation of free fatty acids and elicit neuroprotection in ischemic damage. Increased production of free arachidonic acid and PAF converges to exacerbate glutamate-mediated neurotransmission. These neurotoxic actions may be brought about by arachidonic acid-induced potentiation of NMDA receptor activity and decreased glutamate reuptake. On the other hand, PAF stimulates the further release of glutamate at presynaptic endings. The neuroprotective effects of the PAF antagonist BN 52021 in ischemia-reperfusion are due, at least in part, to an inhibition of presynaptic glutamate release. PAF also induces expression of the inducible prostaglandin synthase gene, and PAF antagonists selective for the intracellular sites inhibit this effect. The PAF antagonist also inhibits the enhanced abundance, due to vasogenic cerebral edema and ischemia-reperfusion damage, of inducible prostaglandin synthase

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mRNA *in vivo*. Therefore, PAF, an injury-generated mediator, may favor the formation of other cell injury and inflammation mediators by turning on the expression of the gene that encodes prostaglandin synthase.

INTRODUCTION

THE UNDERSTANDING OF THE MOLECULAR PATHOPHYSIOLOGY of neurotrauma is providing new clues that may lead to effective therapeutic approaches. The events linking injury mediators that accumulate in neurotrauma with the activation of genes ~~elicited by these mediators~~ may comprise decision pathways that result in neural damage or cell death. Conversely, some mediators may initiate repair-regeneration responses. Fundamental issues are to identify which second messengers accumulate, the signals that trigger their production, and the pathways that, in turn, they affect in the damaged brain or spinal cord. This approach will identify possible targets for drugs that will limit damage, slow cell death, and promote repair.

Within a cell, several signals may be altered in neurotrauma. Because different types of cells are involved in these responses, ~~these elicit~~ a complex array of cell signal transduction events occur. This sequence of pathophysiological events also affects cell-to-cell communication and may result in alterations in synaptic circuitry leading to delayed neurobehavioral disorders such as posttraumatic epilepsy, delayed amnesia, psychosis, and dementia (for review see Gualtiery and Cox, 1991). Complex mechanisms, activated at the onset of brain injury, lead to long-term neurologic deficits (Bazan, 1970, 1990a,b; Bazan and Rodriguez de Turco, 1980; Siesjö, 1988; Siesjö et al., 1992). This knowledge has led to the search for successful therapies (for review see Siesjö, 1992a,b; McIntosh, 1993). To define novel therapeutic targets, important areas of research will be the temporal sequence of signaling events and their consequences for neural cell function.

The results at the onset of ischemic insult to the brain are activation of K^+ conductance, alterations in mitochondrial function and ATP synthesis leading to loss in ion homeostasis, lowered intracellular pH, net influx of Ca^{2+}, and neurotransmitter release (Folbergrová et al., 1990; Siesjö et al., 1991; Ekholm et al., 1992; Katsura et al., 1993). Release of arachidonic acid (20:4) by phospholipase A_2 (PLA_2) and 20:4-diacylglycerol (DAG)/IP_3 by phospholipase C (PLC) are among the membrane lipid-derived signaling systems activated at the onset of ischemia and neurotrauma (Bazan, 1970, 1989; Aveldaño and Bazan, 1975a). Additionally, potassium depolarization and seizures induced by ECS also reversibly activate these degradative pathways (Bazan, 1970; Birkle and Bazan, 1987; Reddy and Bazan, 1987). Recent studies have shown that activation of a specific PLA_2 (Suga et al., 1990) that releases polyunsaturated fatty acids (PUFA) may lead to the hydrolysis of 1-*O*-alkyl, 2-arachidonoyl-glycerophosphorylcholine (alkyl-, 20:4-GPC) with the release of 20:4 and 1-*O*-alkyl-lyso-GPC (lyso-PAF). The latter is further acetylated at the C_2 position leading to the synthesis of 1-*O*-alkyl,2-acetyl-GPC or platelet-activating factor (PAF). This lipid mediator of inflammation and immune responses (Braquet et al., 1987) is also increased in the brain as a consequence of seizures and ischemia (Kumar et al., 1988; Bazan, 1990b). Experimental evidence suggests that PAF may link early lipid responses to neurotrauma with long-term changes through the modulation of protooncogene expression (Bazan, 1990b; Bazan et al., 1993a).

In the present review we discuss (1) the role of glutamate in exitotoxic neuronal damage, (2) PLA_2 activation in brain injury and accumulation of free arachidonic acid, (3) PLA_2 activation and PAF synthesis, (4) arachidonic acid and neuronal function, (5) prostaglandins in neurotrauma, (6) PAF, PAF receptors, and long-term potentiation, (7) PAF in the expression of gene cascades, (8) PAF and PGS-2 induction, and (9) PAF antagonists that preferentially act on cell surface receptors and exert neuroprotection.

GLUTAMATE AND EXCITOTOXIC NEURONAL CELL DAMAGE

Overstimulation of excitatory amino acid (EAA) transmission can result in neural cell injury and death (Olney, 1986; Rothman and Olney, 1986, 1987). Increased release of glutamate in the hippocampus after traumatic injury (Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990) and during ischemia (Benveniste et al., 1984; Meldrum, 1990; Mitani et al., 1990; Christensen et al., 1991) has been reported. Its neurotoxicity is attributed to Ca^{2+} influx into postsynaptic neurons mediated by the *N*-methyl-D-aspartate (NMDA) class of glutamate receptors (Rothman and Olney, 1986; Benveniste et al., 1988; Choi, 1988; Choi and Rothman, 1990; Meldrum and Garthwaite, 1990). Drugs that either reduce the release of glutamate, or block glutamate receptors, protect the brain from excitotoxic ischemic damage (Choi, 1988; Meldrum, 1990; Meldrum et al., 1992). Also, moderate hypothermia reduces presynaptic release of EAAs and protects the brain from neuronal excitotoxic damage (Busto et al., 1989; Kristián et al., 1992).

More recent studies suggest, however, that non-

NMDA receptors, such as the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor (Siesjö, 1988, 1991), the metabotropic quisqualate receptor linked to GTP binding proteins, phosphodiesteratic degradation of PIP_2 and calcium mobilization from intracellular stores (Aleppo et al., 1992), are also involved in glutamate neurotoxic action. Non-NMDA antagonists have been shown to ameliorate brain damage following transient forebrain ischemia (Le Peillet et al., 1992; Arvin et al., 1994; Pulsinelli and Cho, 1992; Pellegrini-Giampietro et al., 1994).

The vulnerability of CA1 neurons in the hippocampus to delayed cell death (Kirino, 1982; Pulsinelli et al., 1982; Siesjö and Wieloch, 1986; Ordy et al., 1993) is thought to be related to their high ratio of NMDA to non-NMDA receptors and a consequential triggering of excess calcium influx through NMDA receptor channels (Rothman and Olney, 1987). Experimental evidence reveals that factors other than increased glutamate release, such as levels of glucose in plasma (Nedergaard, 1987; Siesjö et al., 1993; Dijk et al., 1994) and the "excitotoxic index" [derived from the equation (glutamate) × (glycine)/(GABA)] (Globus et al., 1991), correlate with the selective vulnerability of the CA1 neurons to ischemic damage. Although CA1 and CA3 fields show a similar rise in glutamate concentration during 5 min of ischemia, the latter does not display neuronal degeneration (Mitani et al., 1992). Additionally, during ischemia, glutamate accumulates in areas of the brain spared from ischemic damage (Globus et al., 1990). Glutamate toxicity may also result from its direct inhibition of high-affinity cystine uptake by neuronal cells through the cystine/glutamate antiport (Murphy et al., 1989, 1990). This reduces the availability of cystine for glutathione synthesis, thus limiting the neuronal antioxidant defenses (Ratan et al., 1994a). The interaction of glutamate with glial receptors (Teichberg, 1991) may also affect glial cell metabolism. Alterations in glial calcium homeostasis have been implicated in white matter injury (Waxman et al., 1991). This can be highly relevant in the neurotoxic actions of glutamate because glial cells appear to play an active role, protecting neuronal cells from oxidative stress (Makar et al., 1994). Increased calcium permeation through calcium channels gated by NMDA and non-NMDA glutamate receptors, stimulation of degradative processes that contribute to free radicals production (Traystman et al., 1991), and reduced formation of glutathione will lead to oxidative stress that, in turn, may underlie neuronal apoptotic death (Ratan et al., 1994b; Kerr and Harmon, 1991). Recent studies further support the key protective role of cellular antioxidants (i.e., glutathione) against oxidative stress-induced apoptotic neuronal death (Ratan et al., 1994a). This study reveals that inhibitors of protein synthesis exert neuroprotection from oxidative damage by favoring the conversion of glycine to glutathione.

Calcium permeation through NMDA receptors activates PLA_2 (Lazarewicz et al., 1992a), leading to the generation of membrane phospholipid-derived second messengers and modulators of neuronal function. During early stages of trauma/ischemia, overstimulation of this pathway, due to increased release and accumulation of glutamate, will contribute to neuronal injury. This is discussed in detail in the following section.

PHOSPHOLIPASE A_2 ACTIVATION IN BRAIN INJURY AND ACCUMULATION OF FREE ARACHIDONIC ACID

The activation of PLA_2 is an early event in brain injury and leads to the formation and accumulation of bioactive metabolites (Bazan, 1970). Many of these changes are the result of enzyme activation via an increased calcium influx and/or of intracellular calcium mobilization in the cell (Nicotera et al., 1992). The metabolism of phospholipids in excitable membranes is very sensitive to neurotrauma, cerebral ischemia, or seizure activity (Bazan, 1970; Siesjö et al., 1982; Rodriguez de Turco and Bazan, 1983; Politi et al., 1985; Yoshida et al., 1984, 1986; Birkle and Bazan, 1987; Reddy and Bazan, 1987; Huang and Sun, 1987). At present, the detailed events, such as specific signals involved in the acute sensitivity of neural phospholipases to neurotrauma, are not fully understood.

Under normal conditions, the metabolism of 20:4-containing phospholipids in the CNS, regulated through PLA_2 and acyltransferase, favors the maintenance of very low levels of free 20:4 (Bazan, 1989). During cerebral ischemia, after a lag period following the lowering of oxygen tension in the brain, there is a massive release of free 20:4 (Pediconi and Rodriguez de Turco, 1984; Yasuda et al. 1985; Abe et al., 1987; Sun, 1992; Katsura et al., 1993; Katayama et al., 1994). This lag is when membrane depolarization and calcium influx occur (Katsura et al., 1993). Free 20:4 accumulates gradually as ATP decreases but before membrane depolarization. This could be related to impairments in the activation/acylation pathway, which is sensitive to a decreased ATP/ADP ratio (Katsura et al., 1993).

Two enzymatic pathways have been suggested as contributors to 20:4 release during the rapid early phase of free fatty acids (FFAs) accumulation: PLA_2-mediated deacylation of phospholipids (Bazan, 1970) and the sequential degradation of inositol lipids by PLC-diacylglycerol and monoacylglycerol lipases (for review see Bazan, 1989; Farooqui et al., 1992) (Fig. 1). Also acti-

vation of a phospholipase D (PLD) that hydrolyzes phosphatidylcholine may release phosphatidic acid that, in turn, is acted upon by phosphatidate phosphohydrolase (Farooqui et al., 1992; Nishizuka, 1992), contributing to the enlargement of the DAG pool. Excitable membrane phospholipids contain relatively large proportions of PUFA, 20:4n-6, and docosahexaenoate (22:6n-3), and display phospholipid-hydrolyzing enzymes, i.e., PLA_2, PLC, and PLD (Aveldaño and Bazan, 1975b; Bazan, 1971a,b; Farooqui et al., 1992; Woelk and Porcellati, 1973). Since 20:4 and 22:6 are actively released during seizures and neurotrauma (Bazan, 1976, 1989; Bazan et al. 1981, 1984), it is likely that several PLA_2s are involved in their metabolism. Increased activity of PLA_2 has been reported after ischemic and traumatic injury (Shohami et al., 1989; Rordorf et al., 1991; Bonventre and Koroshetz, 1993), and a plasmalogenase is activated in cerebral ischemia (Edgar et al., 1982).

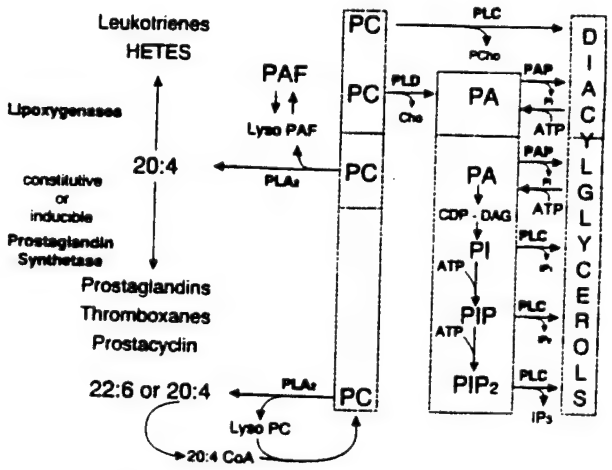

FIG. 1. Phospholipases in the CNS and the generation of lipid-derived second messengers. Under normal physiological conditions, there is a relatively low activity of calcium-dependent phospholipases. Excessive stimulation of ligand-gated calcium channels and the breakdown of energy-dependent calcium homeostasis result in a dramatic rise in intracellular calcium and a resulting overactivation of phospholipases A_2, C, and D. Pools of membrane lipid-derived second messengers are greatly enlarged. PLA_2 action generates 20:4, a substrate for the generation of biologically active eicosanoids and PAF. PLC generates phosphatidic acid (PA), a precursor of inositol phosphate second messengers, and diacylglycerols (DAG), which activate a set of protein kinases. PLD releases choline from PC, thus contributing to the PA pool. These conditions can be self-propagating: the IP_3 receptor gates calcium from the endoplasmic reticulum, thus further raising intracellular calcium levels; and, as discussed later, PAF can induce expression of the inducible prostaglandin synthase and thus further enhance eicosanoid production. Note, however, that under conditions of energy deficit, such as during cerebral ischemia, ATP-dependent processes, such as production of inositol phosphate production and the rephosphorylation of DAG, are inhibited.

The generation of free 20:4 through the PLC-DAG lipase pathway during ischemia and seizures is supported by the observation that free 20:4 accumulates in the brain. This coincides with the release of 20:4-DAG through the phosphodiesteratic degradation of polyphosphoinositides (PPI; Ikeda et al., 1986; Yoshida et al., 1986; Reddy and Bazan, 1987; Abe et al., 1987; Sun, 1992). Dissociation between both lipid effects, FFAs and DAGs, was observed in the anoxic immature murine brain that displays a significant accumulation of free 20:4 after a prolonged lag period, with no accompanying change in 20:4-DAG (Rodriguez de Turco and Bazan, 1983).

Several questions regarding the timing and sequence of phospholipase activation and the origin of membrane-derived lipid mediators remain to be answered. (1) Is the activation of PLA_2 and of PLC simultaneous and/or sequential events? (2) Are both enzymes activated at the same cellular and/or subcellular level? (3) Which mechanism(s) triggers PLA_2 activation and to what extent does the activation of one enzyme (i.e., PLC) contribute to the activation of the other? (4) Which phospholipid pools are the targets for these enzymes?

Analysis of the kinetics of PPI degradation and the parallel accumulation of free 20:4 and 20:4-DAG in rat brain following a single ECS suggests that PLC/PLA_2 activation is time-dependent and that different phospholipids are involved (Reddy and Bazan, 1987; Bazan et al., 1993a). During the tonic phase of the seizure (10 sec after 1 ECS), the decrease of 20:4 from PPI accounts for the accumulation of 20:4 and 20:4-DAG. At later times during the clonic phase (10–30 sec), degradation of other 20:4-phospholipids contributes to the enlargement of the free 20:4 pool. The early accumulation of PPI-derived second messengers appears to involve activation of both PLC and PLA_2. Pretreatment of the rats with *Ginkgo biloba* extract (EGB 761, Tanakan, IPSEN), previously described to elicit neuroprotection during ischemia (De Feudis, 1991), blocks the ECS-induced release of 20:4-DAG in the hippocampus at 10 sec while the accumulation of free 20:4 is not affected (Rodriguez de Turco et al., 1993). The sequential contribution of the PLC/DAG lipase pathway, followed by PLA_2, to the accumulation of free 20:4 in the mouse brain after postdecapitation ischemia has also been reported (Sun, 1992; Sun et al., 1992). Using phenylmethylsulfonyl fluoride, a specific inhibitor of PLC, Umemura et al. (1992) reported that during the first 2 min of complete ischemia in the rat brain, the degradation of inositol lipids via the PLC/DAG-lipase pathway is the main contributor to the release of FFAs. After longer times of ischemia (2–4

min), the calcium-dependent PLA_2 pathway prevails, releasing fatty acids from phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Furthermore, pretreatment with the NMDA antagonist MK-801, which blocks calcium permeation through glutamate receptor channels, also inhibits the PLA_2-mediated degradation of PC and PE. On the same line, kynurenic acid, a broad spectrum antagonist of excitatory amino acids, delays massive hippocampal ionic fluxes and membrane depolarization during ischemia (Mitani et al., 1992) and greatly reduces the accumulation of 20:4 and other free fatty acids within 1–2 min of ischemia (Katayama et al., 1994).

Among the several mechanisms that may contribute to neurotrauma-triggered PLA_2 stimulation are (1) failure in the Na^+-proton antiport and, as a consequence, PLA_2 activation ~~in platelets~~ as proton efflux is activated and localized cytoplasmic alkalinization occurs (Sweatt et al., 1986) and (2) agonist interaction with receptor-G protein complex (Axelrod et al., 1988). Dissociation of the heterotrimeric G proteins into α- and $\beta\gamma$-subunits mediates the activation of phospholipases: the α-subunit stimulates PLC while the $\beta\gamma$-subunits have been shown to activate PLA_2 in retinal rod outer segments (Axelrod et al., 1988; Jelsema and Axelrod, 1987). A recent study reveals high homology between PLA_2 activating protein (PLAP) and β-transducin, further supporting the involvement of the G protein signaling pathway in the direct activation of PLA_2 (Peitsch et al., 1993). A third mechanism is increased cytosolic concentration of $[Ca^{2+}]_I$ triggered either by IP_3-mediated calcium mobilization from intracellular stores (Nicotera et al., 1992; Nishizuka, 1992; Berridge, 1993) or by agonist-receptor-gated calcium channels (Nicotera et al., 1992). The release of glutamate during hypoxia/ischemia leads to the postsynaptic opening of NMDA receptor-coupled Ca^{2+} channels. Intracellular Ca^{2+} overload and stimulation of Ca^{2+}-dependent degradative processes follow, which effect proteins, nucleic acids, and lipids, and mediate glutamate neurotoxicity (Nicotera et al., 1992). PLA_2-mediated release of 20:4 is triggered by NMDA receptor activation in primary cultures of striatal neurons and cerebellar granule cells (Dumuis et al., 1988; Lazarewicz et al., 1988, 1992a) and in hippocampal slices (Pellerin and Wolfe, 1991). PLA_2 inhibitors block the NMDA-dependent release of 20:4 (Sanfeliu et al., 1990; Lazarewicz et al., 1992a). A high-molecular-weight cytosolic $cPLA_2$ that translocates to the membrane when there is an increase in $[Ca^{2+}]_I$ is present in the CNS (Yoshijara and Watanabe, 1990; Bonventre and Koroshetz, 1993). Moreover, this type of phospholipase preferentially hydrolyses 20:4-phospholipids in the brain as well as in other tissues (Fujimori et al., 1992; Kudo et al., 1993).

The release of 20:4 may also be modulated by lipocortins (also known as annexins), a family of calcium and phospholipid binding proteins that inhibits PLA_2 (Flower, 1988). Antiinflammatory properties of glucocorticoids are linked to their ability to induce lipocortin synthesis. The presence of the steroid-inducible protein lipocortin-1 in glial and neuronal cells of the brain, especially in the hippocampus, has been reported (Strijbos et al., 1991). Subsequent studies from the same laboratory show that lipocortin-1 administered intraventricularly to rats significantly reduces the infarct size and edema induced by ischemia (Relton et al., 1991) and attenuates excitotoxic damage mediated by NMDA receptors (Black et al., 1992). Lipocortin-1 is considered to give endogenous neuroprotection against injury and disease (for review see Rothwell and Relton, 1993). Lipocortin-1 was reported to be present in the normal human CNS and increased in diseased CNS mainly associated with invading and resident macrophages (~~Jeruszalinsky et al., 1994; Birkle and Bazan, 1970, 1987, 1994~~). Lipocortin-1, as well as the structurally related lipocortins 2, 4, and 5, has been detected in the CNS of normal individuals postmortem and significantly increased in patients with multiple sclerosis (MS), an inflammatory and demyelinating disease of the CNS (Elderfield et al., 1992). Similar observations were reported in Lewis rats with experimental allergic encephalomyelitis (EAE), an animal model of MS (Elderfield et al., 1993; Bolton et al., 1990). Increased levels of glucocorticoids in plasma from EAE-diseased rats suggest that endogenous steroids may contribute to the observed spontaneous recovery from acute EAE, at least in part, via the steroid-inducible protein lipocortin-1 (Elderfield et al., 1993). Moreover, treatment with antiinflammatory steroids also suppresses EAE (Bolton and Flower, 1989). In a model of rat brain vasogenic edema induced by cryogenic injury, pretreatment with dexamethasone greatly reduces the degree and duration of edema accompanied by lower accumulation of free 20:4n-6 and 22:6n-3 (Politi et al., 1985). Dexamethasone treatment also reduces brain edema generated in rats by stereotaxic injection of PLA_2 into one hemisphere (Heuze et al., 1993). All these studies support the idea that PLA_2-mediated release of 20:4 plays a central role in the development of neuronal injury (Bazán, 1976). Eicosanoids synthesized from free 20:4 are potent mediators of inflammation, increasing membrane permeability and favoring the development of brain edema (Shimizu and Wolfe, 1990). The presence of steroid-inducible lipocortins in the CNS may contribute to the modulation of PLA_2 activity and release of second messengers under both physiological and pathological conditions.

Free 20:4 is directly involved, as it will be discussed in the following section, in the modulation of neuronal

activity, in the pathophysiology of trauma, and in ischemia/reperfusion-induced brain damage. Differences in the release of 20:4 between the CA1 and CA3 areas of the hippocampus during cerebral ischemia have been reported (Westerberg et al., 1987). The former displays higher accumulation of 20:4 correlating to its higher vulnerability to ischemic damage as compared with the CA3 area (Kirino, 1982; Kirino et al., 1992). Also, 20:4 continues to rise following 1 day of reperfusion after 5 min of ischemia in the CA1 area. In the cortex, which is less vulnerable to ischemic damage, the levels of 20:4 recover to basal levels after 30 min of reperfusion (Abe et al., 1992).

ARACHIDONIC ACID AND NEURONAL FUNCTION

Arachidonic acid is involved in the regulation of the activity of a variety of functional membrane proteins, i.e., ion channels, receptors, and enzymes (Volterra et al., 1992; Bazan, 1990a,b; Shinomura et al., 1992; Ordway et al., 1991). In the CNS, arachidonic acid release from excitable membrane lipids plays a physiological role modulating synaptic activity. The various modulatory actions of free 20:4 may be the result of direct interaction of the free fatty acid with proteins and/or indirect effects mediated by its conversion to eicosanoids, a cyclooxygenase- and lipoxygenases-generated oxygenated metabolites (Volterra, 1989; Shimizu and Wolfe, 1990; Piomelli, 1994).

The release of 20:4 as a consequence of NMDA receptor stimulation results in perturbations of ionic homeostasis and alterations in neuronal excitability. Arachidonic acid-mediated activation of K^+ channels leads to neuronal hyperpolarization and synaptic depression (Volterra et al., 1992), while inhibition of glutamate uptake (Yu et al., 1986; Volterra et al., 1992; Chan et al., 1983b; Barbour et al., 1989), stimulation of glutamate release (Williams et al., 1989; Dorman et al., 1992; Lynch and Voss, 1990; Zhang and Dorman, 1993), and activation of A_1-adenosine receptors (Domanska-Janik, 1992) favor neuronal excitability. Also, 20:4 released through NMDA receptor activation has a positive feedback effect at the receptor level as it potentiates NMDA receptor currents by increasing open-channel probability (Miller et al., 1992) (Fig. 2). Analysis of the NMDA receptor amino acid sequence reveals the presence of a domain homologous to fatty acid-binding proteins (Petrou et al., 1993). This supports a direct effect of free 20:4 and other free fatty acids on the modulation of NMDA receptor activity. Arachidonic acid may also stimulate glutamatergic neurotransmission, probably acting as a retrograde messenger at the presynaptic level stimulating further release of glutamate. These pre- and postsynaptic effects may contribute to long-lasting changes in glutamate synaptic efficacy or long-term potentiation (LTP), a form of synaptic plasticity essential to the process of memory and learning in the brain (Teyler and DiScenna, 1984). The role of 20:4 as a retrograde messenger in LTP has been reviewed by Lynch et al. (1993). Experimental evidence supports its role in LTP: (1) free 20:4 is increased in postsynaptic membranes isolated from the dentate gyrus after the induction of LTP (Lynch et al., 1993; Clements et al., 1991); (2) arachidonic acid induces LTP in CA1 neurons of hippocampal slices (Kato et al., 1991); (3) inhibitors of phospholipase A_2 and lipoxygenase block the induction of LTP in dentate and CA1 neurons *in vitro* (Lynch et al., 1989; Williams et al., 1989; Saito et al., 1992; Linden et al., 1987); (4) arachidonic acid, in concert with DAG, activates α-, β-, and [γ]-subspecies of protein kinase C (Shinomura et al., 1992) and stimulation of PKC appears to play a central role in LTP (Shinomura et al., 1992; Lynch et al., 1993).

An enhanced degradation of phospholipids mediated by PLA_2 and PLC early in ischemia may contribute to synaptic vulnerability and damage (Bazan, 1989). In fact, when overstimulated, glutamate neurotransmission involved under physiological conditions in neuronal plas-

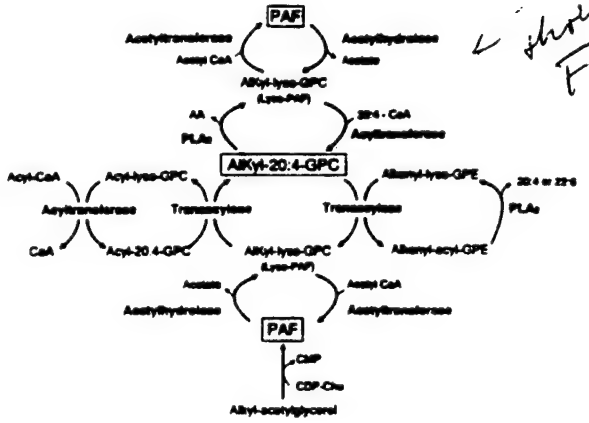

FIG. 2. Enzymatic pathways involved in the PAF cycle. The synthesis of PAF by the "remodeling pathway" involves phospholipase A_2-mediated release of 20:4 from alkyl-arachidonyl-GPC (AAGPC), followed by the acetylation of C_2 by acetyltransferase. Alternatively, PAF may be formed by *de novo* synthesis from alkyacetylglycerol or by a CoA-independent transacylation pathway where AA is transferred from AAGPC to alkenyl lyso-GPE. The enzymatic pathways involved in the "off signal" for PAF action regulated by PAF acetylhydrolase is also shown. The lyso-PAF generated can be reacylated at the C_2 position to generate AAGPC either by an acyltransferase or by a CoA-independent transacylase.

ticity will lead to neuronal degeneration and death (Meldrum and Garthwaite, 1990). 20:4 may also promote neuronal damage by disrupting cellular energy metabolism at the mitochondrial level (Rehncrona et al., 1979; Lazarewicz et al., 1992b; Hillered and Chan, 1988a,b), and by promoting vasogenic brain edema (Chan and Fishman, 1976; Chan et al., 1983a) as a consequence of increased capillary permeability (Unterberg et al., 1987).

PROSTAGLANDINS IN NEUROTRAUMA

The release of 20:4 by PLA_2 is the rate-limiting step in the synthesis of prostaglandins, potent modulators of neuronal function (Shimizu and Wolfe, 1990; Piomelli, 1994). The molecular mechanism of ischemia/reperfusion-induced neuronal injury involves the release of 20:4 during the ischemic phase and its conversion to prostaglandins (Gaudet et al., 1981; Kempski et al., 1987) and leukotrienes (Moskowitz et al., 1984; Dempsey et al., 1986) during reperfusion. These metabolites can elicit a variety of effects, not only in neural cells but also in the microvasculature: prostaglandins, by affecting cerebral blood flow (Moncada and Vane, 1979), and leukotrienes, by increasing blood–brain barrier permeability (Unterberg et al., 1987; Baba et al., 1991).

The rate-limiting enzyme in the synthesis of prostaglandins from free 20:4 is prostaglandin synthase (PGS, prostaglandin endoperoxide synthase, PGH synthase, cyclooxygenase, EC 1.14.99.1), which catalyzes the cyclooxygenation of arachidonic acid to PGG_2 followed by its hydroperoxidation to PGH_2 (Shimizu and Wolfe, 1990). This pathway leads to the formation of free radicals (Kontos et al., 1985) that may contribute to peroxidative damage of neuronal membranes and loss of functional integrity (Hall et al., 1993; Bazan and Rodriguez de Turco, 1980; Siesjö and Katsura, 1992). Two forms of PGS, a constitutive enzyme (PGS-1) insensitive to glucocorticoids and an inducible enzyme (PGS-2) expressed in response to mitogen stimulation and sensitive to glucocorticoid inhibition, have recently been reported (Herschman, 1994; Herschman et al. 1993). PGS-2 was originally cloned as one of a series of primary response genes induced in Swiss 3T3 cells by tetradecanoyl phorbol acetate (TPA), known as TPA-induced sequences (TIS genes). The TIS10/PGS-2 gene is also induced in this cell line by serum and platelet-derived growth factor (O'Banion et al., 1991; DeWitt and Meade, 1993). In the brain, the inducible isoenzyme has been found expressed mainly in cortical and limbic neurons but not in glia or vascular endothelium (Yamagata et al., 1993). NMDA-dependent synaptic activity implicated in memory and learning processes as well as in excitotoxic neuronal damage has been shown to regulate the expression of TIS10/PGS-2 neuronal genes (Yamagata et al., 1993). This implies that prostaglandins can play a role in NMDA-mediated neuronal plasticity and, under pathological conditions (i.e., ischemia, stroke), in neural cell injury and death.

The TIS10/PGS-2 gene is rapidly induced in cells by IL-1 (Diaz et al., 1992; Rzymkiewicz et al., 1994), and its expression is modulated by agents such as transforming growth factor-β (TGF-β; Reddy et al., 1994) and glucocorticoids (Kujubu and Herschman, 1992; O'Banion et al., 1991). The search for new nonsteroidal antiinflammatory drugs that selectively regulate (suppress) its expression while minimally affecting PGS-1 may lead to new antiinflammatory drugs having minimal side effects.

PHOSPHOLIPASE A_2 ACTIVATION AND PAF SYNTHESIS

The synthesis of PAF, another potent mediator of inflammation and immune responses (Braquet et al., 1987), is also stimulated by PLA_2 activation (Bazan, 1990b). The pathways involved in PAF metabolism are summarized in Figure 2. One type of phosphatidylcholine, 1-*O*-alkyl-2 acyl(20:4)-GPC, is a substrate for PAF synthesis through a "remodeling pathway," which involves, in a sequential two-step process, the removal of the acyl chain followed by acetylation of C2. This pathway is very active in inflammatory cells and is involved in cellular responses to external stimuli (Prescott et al., 1990). The calcium-sensitive and arachidonoyl-specific $cPLA_2$ has been shown to regulate PAF synthesis in granulocytic HL-60 cells (Suga et al., 1990). With the use of monoclonal antibodies, $cPLA_2$ has been localized in human cerebral cortex, predominantly in astrocytes. This suggests that these glial cells play an active role in ischemic/traumatic brain injury by generating the inflammatory mediators 20:4 and PAF (Stephenson et al., 1994).

There are at least two other pathways through which PAF is synthesized: the *de novo* pathway involves the transfer of phosphorylcholine to the acceptor alkylacetylglycerol by a choline phosphotransferase insensitive to dithiothreitol (Lee et al., 1986, 1988). This route for PAF synthesis can operate in the CNS (Bussolino et al., 1986; Francescangeli and Goracci, 1989; Baker and Chang, 1993; ~~Bussolino et al., 1988~~), may play a role in the maintenance of physiological cellular levels of PAF (Lee et al., 1986), and seems relevant during the chick retina development (Bussolino et al., 1988). The other route for PAF synthesis involves a CoA-independent transacylation pathway that has been demonstrated in nonneural tissue (Uemura et al., 1991; Ninio et al., 1991;

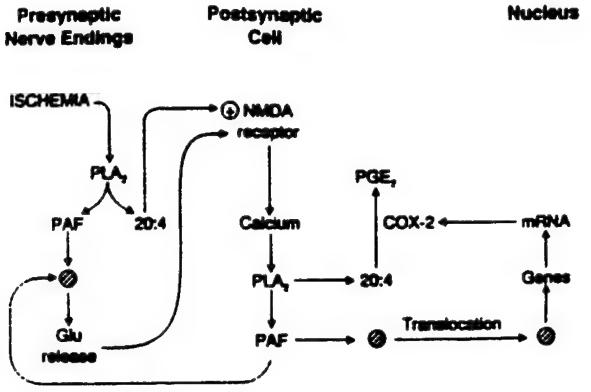

FIG. 3. Traumatic brain injury and ischemia activate signaling mechanisms generated by phospholipase A_2 at the synapse. Brain injury is associated with an increase in free fatty acids in brain tissue as a consequence of PLA_2 activation in pre- and postsynaptic membranes. PAF synthesis may also be stimulated as a consequence of PLA_2 activation. PAF generated at the pre- and/or postsynaptic terminals can interact with presynaptic receptors and stimulate further release of glutamate. In turn, glutamate interacts with NMDA receptors leading to calcium influx, PLA_2 activation, release of AA, and PAF synthesis. The PAF antagonist BN 52021, selective for the presynaptic receptor, elicits neuroprotection from ischemia/reperfusion brain damage, decreasing the accumulation of FFA (Panetta et al., 1987). PAF turns gene expression on (Bazan et al., 1991) through a mechanism that is inhibited by the PAF antagonist BN 50730, selective for intracellular binding sites (Marcheselli and Bazan, 1994). This site may be in the path leading to the stimulated transcription of the gene that encodes the inducible prostaglandin synthase (TIS10, PGS2, COX-2). As a result, PGE_2 is formed.

Venable et al., 1991; Colard et al., 1993; Blank et al., 1995), and in brain (Blank et al., 1995). A transacylase highly selective for polyunsaturated acyl groups (i.e., 20:4, 22:6) transfers the acyl chain from the 1-*O*-alkyl-2-acyl-GPC to a lysophospholipid acceptor alkyl-1-enyllyso-glycero-phosphorylethanolamine (GPE) leading to the formation of the intermediate lyso-PAF (Blank et al., 1995). This mechanism requires the release of the unsaturated fatty acid from C2 of the ethanolamine plasmalogen by a PLA_2. Although this cycle has yet to be evaluated in neurotrauma, the plasmalogenase activity described by Edgar et al. (1982), could be involved. Its increased activity during ischemia could favor, through the formation of alkyl-lyso-GPE, the shuttle of 20:4 from PC plasmalogen to PE plasmalogen and the release of lyso-PAF for PAF synthesis.

PAF is rapidly inactivated by the enzyme PAF acetylhydrolase, a PLA_2 that selectivity cleaves short acyl chains at the C2 position, producing the inactive lyso-PAF. This enzyme is present in brain (Hattori et al., 1993) and other tissues (Farr et al., 1980; Blank et al., 1981).

The resynthesis of 1-*O*-alkyl-2-acyl-GPC can be accomplished by the CoA-dependent acylation of lyso-PAF, a pathway described in both neural and nonneural tissues (Fleming and Hajra, 1977). Transacylases have also been implicated in PC plasmalogen resynthesis from lyso-PAF in platelets and macrophages, where they catalyze the transfer of PUFA from diacyl-GPC to lyso-PAF (Kramer et al., 1984; Sugiura et al., 1987).

The remodeling pathway, rather than *de novo* synthesis, is more likely to be involved in the generation of PAF during CNS trauma, because ischemia promotes PAF accumulation in brain (Kumar et al., 1988) concurrent with elevated intracellular Ca^{2+} levels (Bazan, 1992). However, although PAF, free 20:4, and free 22:6 accumulate in neural trauma and ischemia, it has not been demonstrated that they originate through the activation of the same PLA_2. Several PLA_2 isoforms have been characterized and cloned in mammalian tissues: Type I, Type II, and $cPLA_2$ (Hirashima et al., 1992; Kudo et al., 1993; Yang et al., 1994). It is possible that more than one kind of PLA_2 is activated during brain injury and, in turn, several distinct molecular species of phospholipids can be hydrolyzed. It is also possible that the plasmalogenase-mediated release of 20:4 and 22:6 from PE plasmalogen precedes the formation of PAF through the transacylation pathway.

It is not fully known which cell types in the brain are responsible for trauma-induced PAF synthesis. Because seizures trigger the accumulation of PAF (Kumar et al., 1988) and FFAs (Bazan, 1970), it has been suggested that, at least in the early phase of production of these lipids, they originate from excitable membranes (Bazan, 1990b). Synaptosomal membrane lipids are highly enriched in the PUFA (i.e., 20:4 and 22:6; Cotman et al., 1967; Sun and Sun, 1972), which shows the greatest enrichment in the FFA pool during seizures (Bazan, 1976; Bazan et al. 1981, 1982). Stimulation of synaptosomes *in vitro* results in PLA_2 activation and preferential release of 20:4 and 22:6 (Bradford et al., 1983; Baker and Loh, 1990). Similarly, *in vivo* synaptic activity may activate the PLA_2 pathway with the consequent release of PUFA from these excitable membranes. Isolated synaptosomes from rats undergoing bicuculline-induced status epilepticus show selective increases in the pool size of free 20:4 and 22:6 relative to other free fatty acids (Birkle and Bazan, 1987). Furthermore, these changes were seen 4 min after seizure induction in synaptosomes but not other subcellular fractions.

PAF, PAF RECEPTORS, AND LONG-TERM POTENTIATION (LTP)

Accumulation of membrane-derived second messengers, such as 20:4 and PAF, during neuronal cell injury

is the result of overactivation of processes that normally modulate synaptic function. They can mediate the cellular response to injury leading to repair and/or to cell damage, (Bazan, 1994) probably depending on their cellular origin and impairment in neuronal activity. In fact, the lipid mediator PAF accumulates in the brain during seizures and ischemia (Kumar et al., 1988) and has been linked to ischemia/reperfusion brain damage (Panetta et al., 1987). Its central role as a mediator of inflammatory and immune responses is well recognized (Braquet et al., 1987).

Recent studies show several important physiological roles played by PAF: its ability to stimulate sprouting in PC12 cells when present at low concentrations (Kornecki and Ehrlich, 1988), its stimulatory effect on glutamate release (Clark et al., 1992), and a possible role as a retrograde messenger in long-term potentiation (Kato et al., 1994). To accomplish these effects, PAF interacts with specific binding sites present in the CNS (Bito et al., 1992; Domingo et al., 1988; Thierry et al., 1989; Faden and Halt, 1992). There are also PAF binding sites in hippocampus (Marcheselli and Bazan, unpublished observations) and in transformed neural cells (Squinto et al., 1989, 1990; Marcheselli et al., 1990). Further analysis of the site(s) of PAF action in the brain was pursued at subcellular level in microsomal and synaptic ending membranes isolated from cerebral cortex (Marcheselli et al., 1990) and hippocampus (Marcheselli and Bazan, unpublished observation). The PAF antagonist BN 52021 competes for receptors that are found in the presynaptic membranes (Marcheselli et al., 1990; Bazan et al., 1991). The PAF bioactivity toward these sites is to enhance excitatory neurotransmitter release and the antagonist BN 52021 blocks this effect (Clark et al., 1992; Bazan et al., 1993b). Two PAF binding sites have been characterized in microsomal membranes, one of which displays the highest affinity reported to date (Marcheselli et al., 1990). The intracellular binding sites differ from the extracellular receptor in that they display higher affinity for PAF binding and are antagonized by BN 50730, but not BN 52021 (Marcheselli et al., 1990). Differences in the density of PAF binding sites among brain areas (Bito et al., 1992; Domingo et al., 1988) and the selectivity of antagonists for intra- and extracellular binding sites as well as differences in the kinetics of PAF binding (Marcheselli et al., 1990) have provided new insight into mechanisms involved in the multiple effects elicited by PAF on cell function. This also could explain the susceptibility of different neuronal populations to PAF-mediated neurotoxic and/or plasticity responses.

A cell surface PAF receptor has been cloned (Honda et al., 1991; Nakamura et al., 1991; Ye et al., 1991), and its sequence predicts seven putative transmembrane domains characteristic of receptors linked to G proteins. The identity of the intracellular PAF receptor, as well as its possible relationships with the cell surface receptor, has not been defined. The presence of microsomal binding sites and PAF's transcriptional activating properties (Bazan et al., 1991; Bazan and Doucet, 1993) suggest that stimulus-evoked PAF synthesis may be involved in gene expression and long-term cellular responses (discussed in further detail in the following section).

PAF is involved in the development of long-term potentiation (Del Cerro et al., 1990; Arai and Lynch, 1992; Bazan et al., 1993b; Wieraszko et al., 1993), considered as a model of mechanisms underlying memory and learning in the brain (Teyler and DiScenna, 1984). PAF may be a retrograde messenger in CA1 hippocampal long-term potentiation (Kato et al., 1994). Its effect is mediated by presynaptic receptors because BN 52021 blocks PAF-induced long-term potentiation (Kato et al., 1994). Cultured hippocampal neurons have been used to study the involvement of the presynaptic site of PAF action in the modulation of excitatory amino acid release (Clark et al., 1992). Methyl carbamyl-PAF (mc-PAF), a nonhydrolyzable PAF analog, increased glutamate-mediated evoked excitatory synaptic transmission and enhanced the frequency of spontaneous miniature excitatory synaptic events without modifying their amplitude or their time course. The biologically inactive PAF metabolic intermediate, lyso-PAF, did not affect synaptic transmission. Moreover, mc-PAF effects are likely mediated by the PAF receptor found in presynaptic membranes, because the antagonist BN 52021, which competes with PAF for these binding sites (Marcheselli et al., 1990), inhibits the effect of 1 μM mc-PAF.

In summary, PAF's interaction with presynaptic receptors modulates excitatory amino acid release and may play a role, under normal conditions, in glutamate-mediated synaptic plasticity and, under pathological conditions, in excitotoxicity, seizure generation, and neuronal damage.

NEUROPROTECTIVE ACTION OF PAF ANTAGONISTS THAT ACT PREFERENTIALLY ON PRESYNAPTIC RECEPTORS

PAF antagonists display neuroprotective properties in ischemia/reperfusion damage and in other related experimental models by restoring cerebral blood flow, enhancing survival, and reducing the progression of neuronal damage (Kochanek et al., 1987; Spinnewyn et al., 1987; Prehn and Krieglstein, 1993; Frerich and Feuerstein, 1990). BN 52021 systemically injected into

gerbils at the onset of the reperfusion after 20 min of complete cerebral ischemia restores cerebral blood flow, decreases phospholipid degradation by PLA_2, and elicits overall neuroprotection (Panetta et al., 1987). In spinal cord ischemia/reperfusion, PAF levels are increased 20-fold and the PAF antagonist BN 50739 prevents postischemic hypoperfusion as well as edema formation (Lindsberg et al., 1990). Additionally, BN 52021 and WEB 2170 also exhibit neuroprotective properties in percussive traumatic brain injury (TBI). Behavioral deficits are reduced, edema is limited, and the traumatic content of glycine in the ipsilateral hippocampus is lowered (Faden and Tzendzalian, 1992). The PAF antagonist BN 52021 competes for binding in presynaptic membranes obtained from cerebral cortex (Marcheselli et al., 1990) and hippocampus (Marcheselli and Bazan, unpublished observations), inhibits the stimulatory PAF effect on glutamate release when added to hippocampal neurons in culture (Clark et al., 1992), to *in vitro* perfused retinas, and synaptosomal preparations (Marcheselli and Bazan, 1992), and blocks the excitotoxic damage induced by glutamate in culture of neurons from chick embryos in the absence of glial and endothelial cells (Prehn and Krieglstein, 1993) These data indicate that PAF can be generated and have a direct effect on neurons by interacting with binding sites located in synaptic membranes. Moreover, these experimental evidences also suggest that excitatory amino acid neurotransmission and excitotoxic neuronal damage involve PAF as an agonist. Therefore, the neuroprotective effect of BN 52021 in ischemia/reperfusion (Panetta et al., 1987) may be due, at least in part, to an inhibition of the PAF presynaptic binding sites (Bazan and Cluzel, 1992; Clark et al., 1992).

Excitatory amino acid neurotoxicity appears to be a component of several chronic degenerative diseases of the CNS (Choi, 1988). PAF can itself be neurotoxic by exacerbating glutamate neurotransmission and, consequentially, mediating progressive neuronal damage and loss. For instance, PAF appears to be the neurotoxin that mediates neuronal damage during bacterial meningitis (Cabellos et al., 1992) and in the neurological complications in human immunodeficiency virus type 1 (HIV-1)-infected patients (Gelbard et al., 1994). In the latter study, the authors also show that PAF induces cellular death when added to primary human fetal cortical or rat neurons in culture at concentrations similar to that reported in the CSF of HIV-1 patients. The authors also show that PAF neurotoxicity is blocked by the NMDA receptor antagonists MK-801 and memantine. These results give further support to the idea that PAF, by interacting with presynaptic binding sites, stimulates glutamate release and NMDA receptor-mediated neurotoxicity, leading to neuronal disfunction and eventually to cell death.

Excess calcium influx (Nicotera et al., 1992) and its subsequent sequestration by intracellular organelles, i.e., mitochondria and nucleus, plays a central role in glutamate toxicity. This may stimulate degradative processes leading to alterations in subcellular structures and functions (Eimerl and Schramm, 1994). In a recent study, Sun and Gilboe (1994a) reported increased levels of polyunsaturated fatty acids, mainly 20:4 and docosahexaenoate, in mitochondria isolated from the rat brain during ischemia and reperfusion. This was paralleled by increased free radical-mediated lipid peroxidation and impaired mitochondrial function. When animals were treated with the extracellular PAF antagonist BN 50739, administered at the onset of recirculation, lipid changes were reversed and mitochondrial respiration improved (Sun and Gilboe, 1994b). PAF actions are intimately linked to and mediated by increased cytosolic calcium concentration, the result of either (1) its receptor-mediated activation of PIP_2 phosphodiesteratic degradation with the release of IP_3 that underlies the subsequent mobilization of calcium from intracellular stores (Catalan et al., 1992; Yue et al., 1992), (2) its activation of calcium channels leading to calcium influx (Kornecki and Ehrlich, 1988), or (3) its indirect, NMDA-mediated permeation of calcium (Clark et al., 1992). The search for new, more efficient and selective PAF antagonists with minimal side effects will favor the development of new therapeutic strategies for the treatment of delayed neurological deficits and neurobehavioral disorders triggered by brain injury. These strategies could also delay and/or lessen the severity of inflammatory and chronic degenerative diseases of the CNS.

Neuroprotection elicited by PAF antagonists may include their effect on nonneural cells, such as endothelial cells of the microvasculature and components of the circulatory system. These could prevent PAF-mediated disruption of the blood–brain barrier (Kumar et al., 1988; Sun et al. 1992), vasoconstriction and reduced cerebral blood flow (Kumar et al., 1988; Edwards et al., 1991; Kochanek et al., 1987), and infiltration of leukocytes (Frerich and Feuerstein, 1990), and would favor the reestablishment of blood flow and oxygen delivery to neuronal cells.

PLATELET-ACTIVATING FACTOR AS A MODULATOR OF GENE EXPRESSION

Identification of second messengers and intracellular sites that couple stimulation with gene transcription is a central issue in the study of cell signaling. The rapid and transient expression of immediate-early genes is triggered by many different signals. In the nervous system, physi-

ological and pathological events (such as ischemia, seizures, NMDA receptor activation, and long-term potentiation) initiate transcription and translation of these genes (Morgan and Curran, 1991; Doucet et al., 1990; Doucet and Bazan, 1993; Bazan and Doucet, 1993; Herschman, 1991). Many immediate-early genes encode transcription factors that may elicit cascades of secondary and tertiary gene expression, the means by which long-

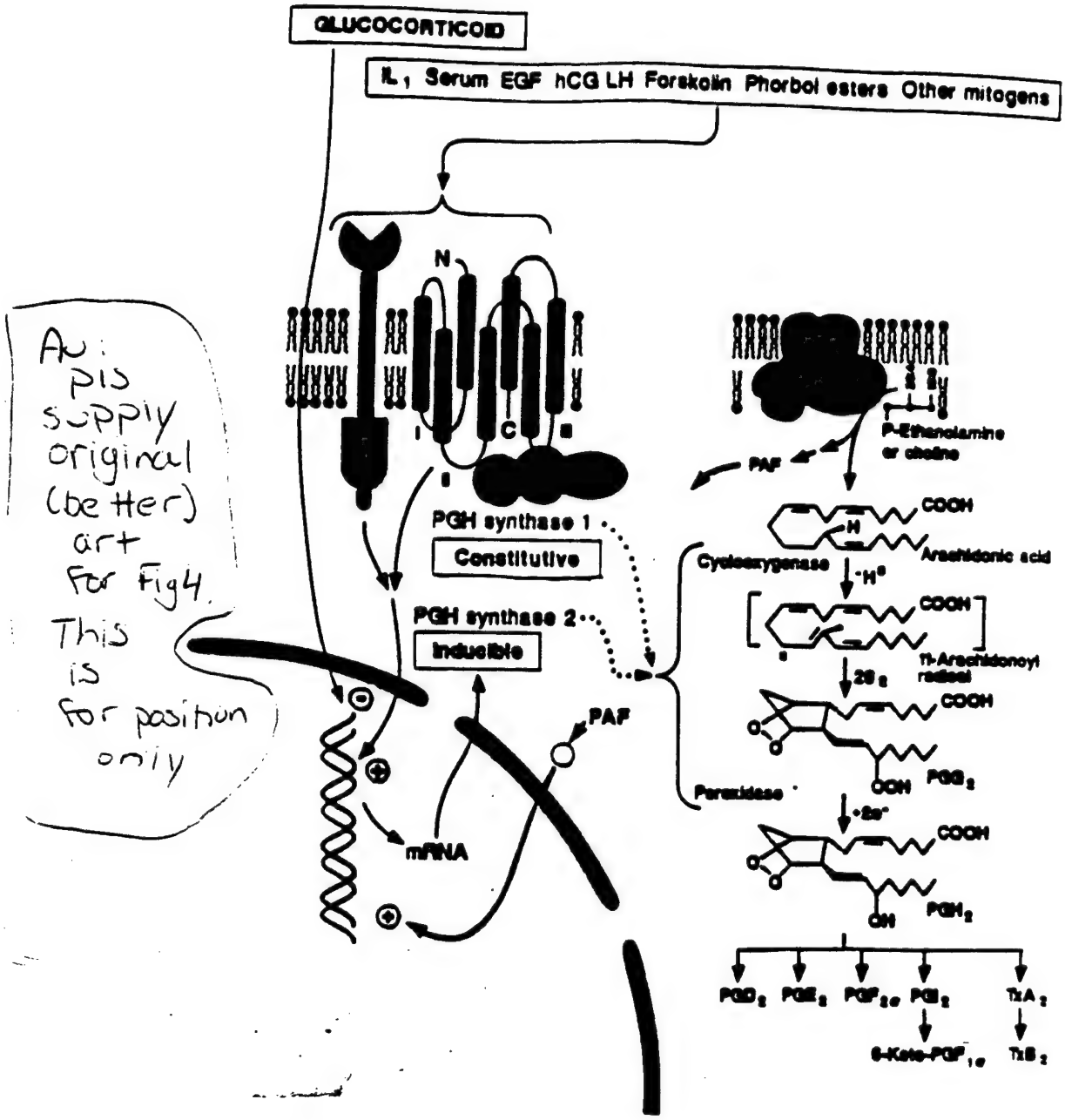

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FIG. 4. Prostaglandin synthase isoenzymes 1 and 2: signals that activate the inducible enzyme (PGS2). Outline depicting mitogens, growth factors, and other activators of receptor-tyrosine kinase and "serpentine" - G protein-linked receptors leading to PGH synthase 2. This inducible isoenzyme 2 catalyzes the cyclooxygenation and peroxidation of arachidonic acid toward PGH2 which, in turn, generates various prostaglandins. The other enzyme that catalyzes this reaction is the constitutive PGH synthase 1. On the right-hand side of the outline, a membrane phospholipid, phosphatidylcholine or phosphatidylethanolamine, is shown to be degraded, yielding arachidonic acid and PAF. This reaction is catalyzed by phospholipase A_2. PAF is depicted to act on a perinuclear site that subsequently activates the inducible PGH synthase 2. Glucocorticoids are shown as inhibitors of this gene.

term cellular responses such as neuronal plasticity can take place. PAF is also able to activate another type of immediate early gene, the inducible prostaglandin synthase (TIS 10/PGS2), which in turn generates more eicosanoid lipid mediators. This is discussed in more detail in a later section.

There is evidence that PAF is a mediator of signal transcription coupling that, in turn, may be involved in neuronal plasticity, remodeling of synaptic circuitry, and epileptogenesis (Fig. 4). PAF rapidly and transiently augments levels of the c-*fos* and c-*jun* transcription factor mRNAs, and phorbol esters and PAF synergistically stimulate c-*fos* expression, suggesting that the transcriptional effects of PAF are not mediated by protein kinase C (Squinto et al., 1989). PAF's effect is at the transcriptional level (Squinto et al., 1989) and 5′ deletion mutagenesis studies of the c-*fos* promoter show that the calcium-response element is necessary for the PAF-induced response (Squinto et al., 1989). PAF activates the expression of c-*fos* and c-*jun* in corneal organ culture (Bazan et al., 1993c). The hetrazepine BN 50730, an antagonist selective for the intracellular PAF binding site, inhibits c-*fos*, c-*jun*, and *zif*-268 expression in several transformed (Squinto et al., 1989, 1990; Bazan et al., 1991; Schulman et al., 1991; Tripathi et al., 1991; Mazer et al., 1991) and primary cells (Bazan et al., 1994) in culture. Intraperitoneal or intracerebroventricular injection of BN 50730 (Marcheselli and Bazan, 1994) inhibits c-*fos* and *zif*-268 expression triggered by electroconvulsive shock in hippocampus (Marcheselli and Bazan, 1992).

PLATELET-ACTIVATING FACTOR AND PROSTAGLANDINS: INDUCIBLE PROSTAGLANDIN SYNTHASE

Although 20:4 and PAF modulate distinct cellular functions, their synthesis and some modes of action may be related. Both can be generated by PLA_2 action on the same membrane lipid precursor, 1-*O*-alkyl-2-arachidonoyl-GPC, and both accumulate in the brain during seizures and ischemia (Bazan, 1990b). By different mechanisms, PAF and 20:4 contribute to glutamate elevation and neurotoxicity, the former by stimulating its presynaptic release (Clark et al. 1992; Bazan et al., 1993b) and the latter by inhibiting its uptake by synaptosomes and astrocytes (Volterra et al., 1992) and potentiating NMDA-receptor currents (Miller et al., 1992). A PAF-mediated increase in intracellular calcium (Kornecki and Ehrlich, 1988; Yue et al., 1991) can also activate PLA_2 and further stimulate 20:4 release in the brain (Kunievsky and Yavin, 1992). This suggests that at least some of PAF's actions as a modulator of neuronal functions can be mediated and/or complemented by 20:4.

In addition, there is now new evidence that receptor-mediated PAF activity may also be involved in the metabolism of 20:4 to eicosanoid lipid mediators. In neural and nonneural cells, PAF and retinoic acid can synergistically induce expression of reporter genes from transfected contructs containing portions of the TIS10/PGS-2 promoter (Bazan et al., 1994). Increases in inducible prostaglandin synthase mRNA in hippocampus triggered by *in vivo* cryogenic injury and ischemia-reperfusion are inhibited by a PAF antagonist selective for intracellular PAF binding sites (Bazan et al., 1991). In a four-vessel occlusion model of cerebral ischemia in the rat, a PAF antagonist inhibits the expression of the inducible prostaglandin synthase in hippocampus. (Marcheselli and Bazan, unpublished observations). It remains to be determined if TIS10/PGS-2 induction in these animal models is part of the injury/inflammatory response or a pathway leading to cell death, or, conversely, if it leads to repair.

Since PAF may affect gene expression through an intracellular site (Squinto et al., 1989; Bazan et al., 1991), its effects in combination with retinoic acid, another lipid-like molecule, which modulates gene expression through well-characterized nuclear receptors, were studied on constructs of the TIS10/PGS-2 promoter with a luciferase reporter transfected into neuroblastoma cells. When the calcium phosphate coprecipitation transfection procedure was used, there was a PAF-dependent (1–50 n*M*) activation of the promoter in the presence of retinoic acid. The intracellular PAF antagonist BN 50730 inhibited this effect, implying that a PAF receptor was involved in the expression of the inducible prostaglandin synthase. The effect of PAF in the presence of retinoic acid is rapid under the conditions of these experiments, suggesting that a preexisting latent transcription factor(s) is engaged in the effect. Deletion studies of the TIS10/PGS-2 promoter/luciferase reporter constructs showed that deletion of sequences between −371 and −300 bp reduced the effect of PAF from 31-fold to 4.1-fold. This reduction was inhibited by BN 50730 when incubated with cultured cells for 1 h before transfection. This suggests that a major PAF response element lies in this region. Further upstream deletions abolished PAF-induced promoter activation.

Prostaglandins generated by neuronal inducible PGS-2 have been implicated in neuronal plasticity (Yamagata et al., 1993). Also, prostaglandins are cytoprotective against various forms of injury in nonneural cells (Robert et al., 1979; Paller and Manivel, 1992) and in the embryonic retinal cell (Dymond and Kalmus, 1992) as well as against glutamate-induced injury to cortical neurons

in primary cultures (Cazevielle et al., 1994). Therefore, PAF, by turning the inducible prostaglandin synthase on in brain, may open a pathway involved in the modulation of synaptic function and neuroprotection, limiting neuronal damage (Fig. 5).

NEUROTRAUMA, INJURY AND BIOACTIVE LIPIDS: OVERALL CONCLUSIONS

The effects of neurotrauma on excitable membranes, as well as other neurochemical and functional changes, are reflected in the accumulation of the membrane-derived lipid second messengers. This accumulation in the synapse during injury represents an overactivation of processes that regulate normal synaptic function. It may play a central role in various pathways leading to brain damage. However, some of these mediators may also activate cellular routes leading to plasticity responses and repair.

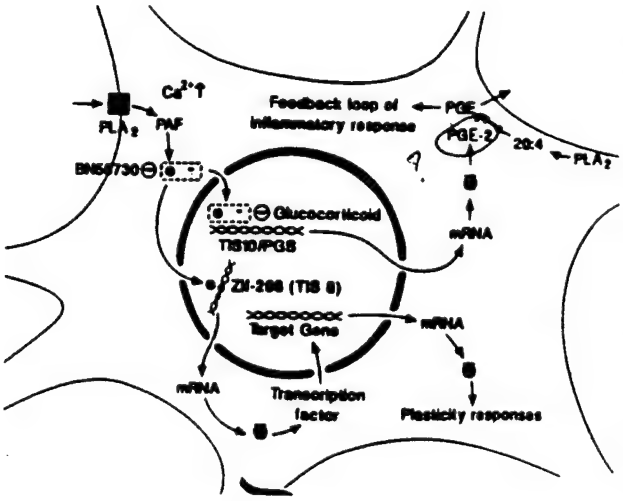

FIG. 5. Ischemia reperfusion-induced gene expression in brain. Calcium and PLA_2 (phospholipase A_2) increase PAF, which, in turn, turns on gene expression through a mechanism that is inhibitable by BN 50730. This PAF antagonist is selective for intracellular PAF binding sites (Marcheselli et al., 1990). Recent evidence suggests that this site may be in the path leading to the TIS/PGS gene (inducible prostaglandin synthase). As a result, PGE_2 from arachidonic acid is formed. This prostaglandin may comprise a feedback loop of the injury-inflammatory response and also elicit other effects. The intracellular PAF binding site leads to the transcriptional activation of other immediate-early genes, such as *Zif*-268 (TIS 8, Egr-1, NGFI-A). The transcription factor encoded by this gene may lead to the expression of late response genes involved in adaptive or plasticity responses.

There is a network of complex interactions between different neurons and other cell types. There are, however, predominant pathways. First, some signals converge to promote excitotoxicity by enhancing glutamate release through PAF, and/or other mediators, acting on the presynaptic ending. These, in turn, overactivate glutamate receptors (e.g., NMDA) and result in increased Ca^{2+} with the activation of degradative enzymes. An early indicator of the degree of cerebral ischemia is the accumulation of free arachidonic and docosahexaenoic acids. Membrane phospholipid breakdown, reperfusion, and other factors contribute to the initiation of free radical reactions that include lipid peroxidation. Moreover, oxidative stress plays a major role in limiting neural cell survival in stroke. Second, there are multiple mediators of the inflammatory response generated. Third, some neurons die early after a cerebrovascular insult, some recover, while others exhibit delayed apoptotic death as a consequence of some initial events. Lastly, it is likely that there are signals that activate plasticity and repair pathways that, in turn, lead to the reestablishment of synaptic circuitry.

Following stroke, there is sometimes remarkable recovery weeks after an initially severe neurological impairment. This may be due to resolution of the injury-inflammatory condition, active neural plasticity that reestablishes some damaged synaptic circuitry, or remodeling of extracellular matrix components important for blood–brain barrier function and for intercellular relations. Some membrane-derived lipid second messengers generated as acute effects of injury may participate in the coupling of injury either with repair/regenerative responses, or with cell death via the activation of gene cascades, as discussed above.

Several physiological mediators in the nervous system may also play roles in pathological conditions. Glutamate, an excitatory amino acid, is, by far, the most abundant neurotransmitter of the mammalian brain and plays a critical role in developmental plasticity, memory formation, etc. However, in neurotrauma and some neurodegenerative diseases, glutamate exhibits abnormally high accumulation, making it a critical effector of excitotoxic damage. Other mediators, such as interleukin-1 and, perhaps, amyloid peptide, also coexist with neural cells under physiological conditions, but may, like PAF, engage in abnormal actions in diseases when overproduced. Although PAF is most often referred to as a mediator of the inflammatory and immune responses and of cell injury, low PAF concentrations elicit sprouting in PC12 cells. At high concentrations, neuropathological changes occur. The presynaptic PAF binding site ([illegible] et al., 1994) linked to glutamate release is a target through which PAF participates in excitotoxicity. However, the

same presynaptic site involves PAF as a potential retrograde messenger in long-term potentiation.

When stimulated by PAF, the inducible prostaglandin synthase gene (PGS-2/TIS-10) represents a link between ischemia-generated PAF and the gene encoding the enzyme that catalyzes the cyclooxygenation of arachidonic acid. Prostaglandin synthase isoenzymes in the brain, as well as their physiological significance and pathological role, is only beginning to be studied. Because PAF synthesis is also activated by glutamate/NMDA interaction, it is possible that, by turning the inducible prostaglandin synthase on in brain, a pathway may open that is involved in modulation of synaptic function. The role played by PAF in the "decision pathways" is not clearly understood. These pathways lead to cellular function, apoptotic death, or, conversely, to survival and plasticity/repair through PAF's stimulatory effect in gene expression followed by neuronal cells after neurotrauma. Inducible prostaglandin synthase in neurotraumatic injury may also be involved in nonneuronal events. Prostaglandins are important modulators of the cerebral circulation and, during reperfusion, there are vasodilation and an enhancement of the cerebral blood flow.

Studying the sequence of events following neural trauma and understanding their consequences help define temporal windows during which pharmacological intervention can limit and even reverse long-term damage. Lipid mediators such as PAF and arachidonic acid metabolites are clearly very important therapeutic targets, and research into designing drugs to modify and control their actions is continuing.

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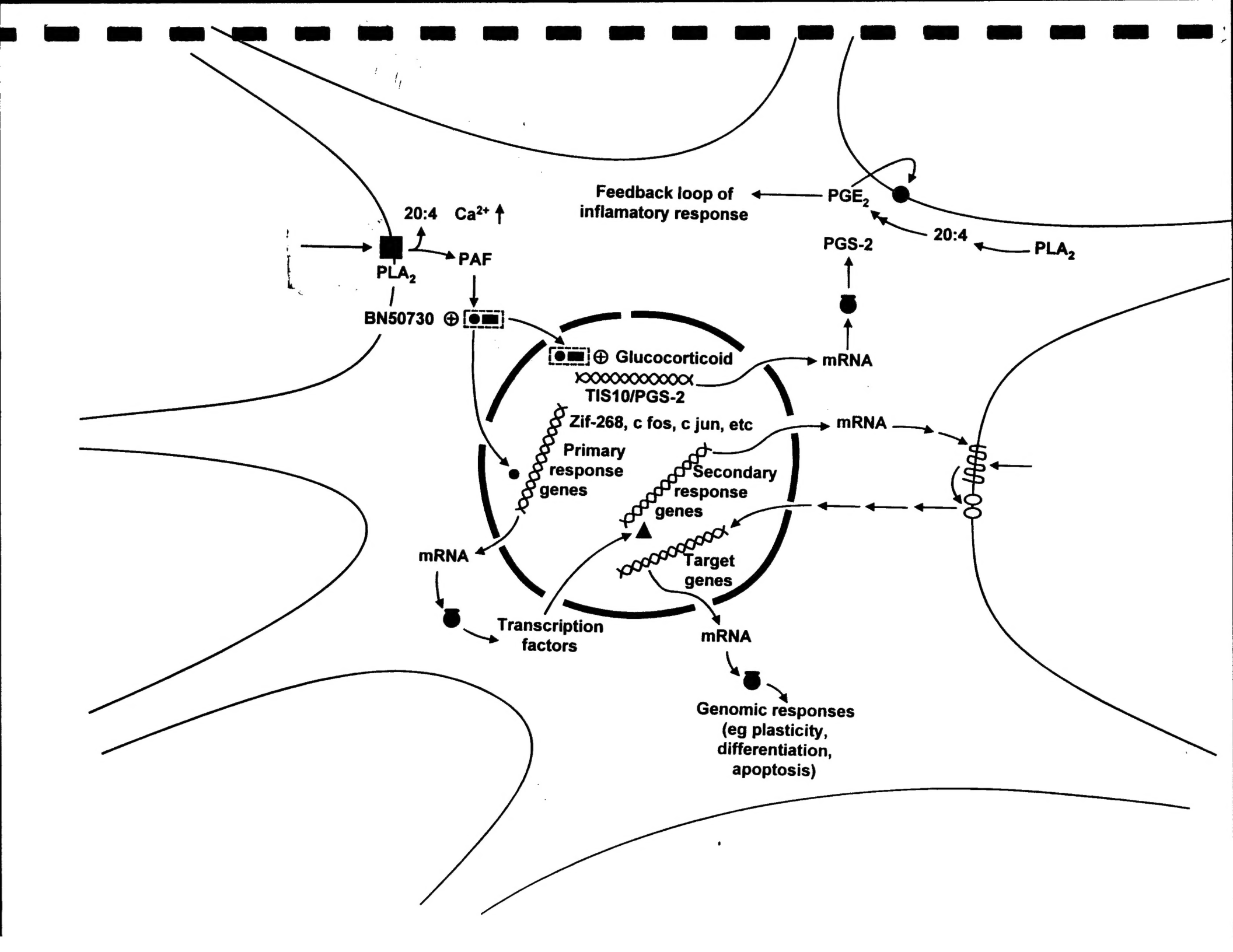
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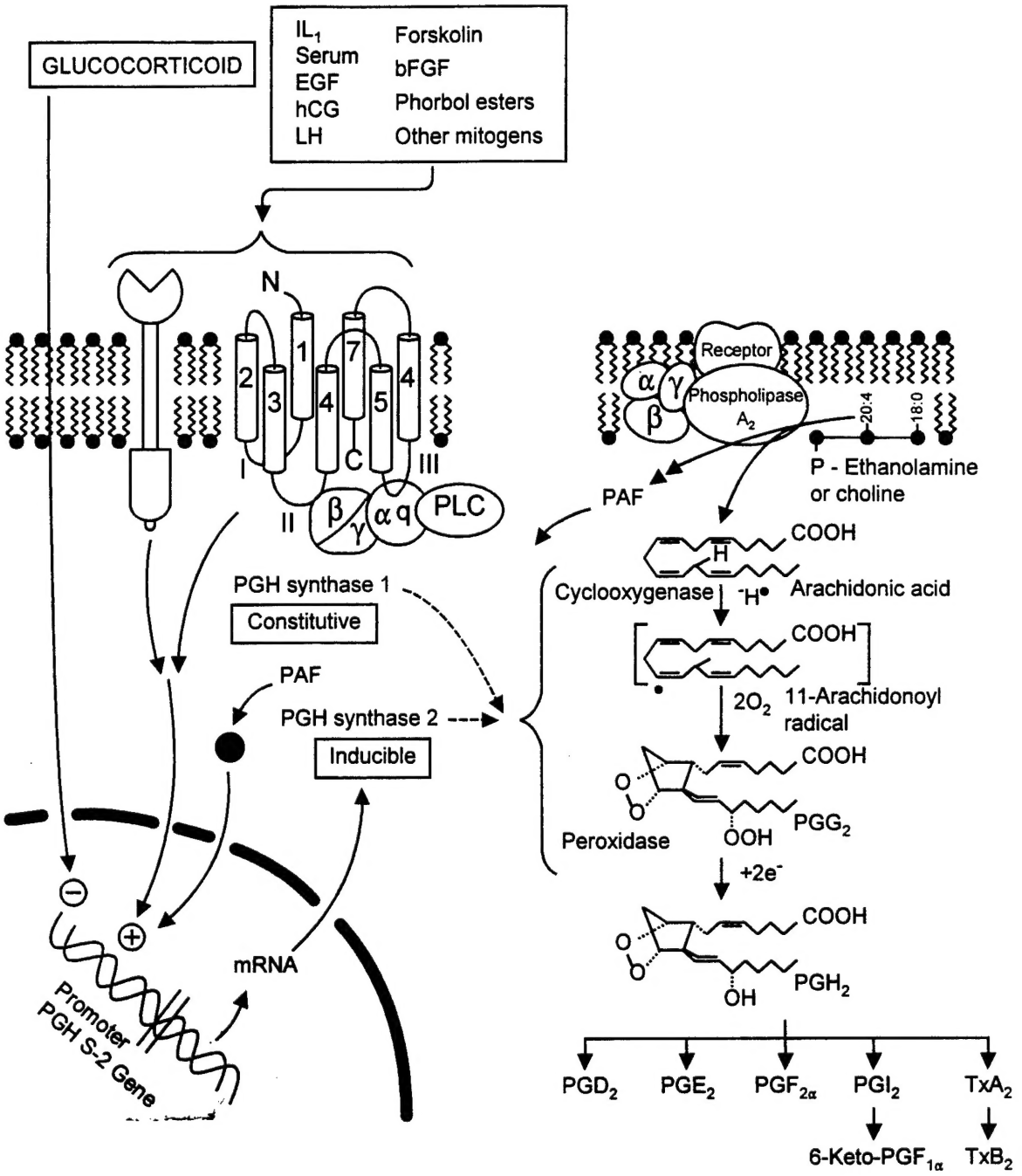
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